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REVIEW

REVIEW OF GAS-LIQUID CHROMATOGRAPHY

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During the last few years gas chromatography has become established as a rapid, efficient, and relatively simple technique for the separation and analysis of mixtures of volatile substances. The present review is intended to survey critically the large amount of published work on theoretical principles, apparatus, techniques, and applications, and to give a comprehensive bibliography on all aspects of gas-liquid chromatography.

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DEFINITIONS AND HISTORICAL

The recommendations of the Hydrocarbon Research Group of the Institute of Petroleum [580] will be followed in this review. The term "gas chromatography" will be used for all those chromatographic techniques in which a mobile gas phase carries the substances to be separated through a stationary phase packed into a suitable container. When the stationary phase is a solid adsorbent, the method is Gas–Solid Chromatography (G.S.C.); when the stationary phase is an absorbent liquid supported by inert material, the method is Gas–Liquid Chromatography (G.L.C.).

The first gas chromatographic experiments were carried out by selective adsorption on, or desorption from, solid adsorbents such as active charcoal. This basic method is of long standing, for RAMSAY [500] used it in 1905 to separate mixtures of gases and vapours. CLAESSON [101] has given a detailed bibliography of the literature up to 1944 and JANAK [328] has recently reviewed G.S.C. in detail.

Following the theoretical suggestion of MARTIN AND SYNGE [412], the method of G.L.C. was introduced by JAMES AND MARTIN [314] in 1952. This made possible the separation and estimation of small amounts of volatile substances with a very wide range of boiling points, and thus gave a great impetus to many fields of research.

SECTION I. PRINCIPLES INVOLVED IN SEPARATIONS BY G.C.

Methods of operating columns

In G.C. elution analysis, frontal analysis, and displacement development can be used. For elution analysis the mixture of substances to be separated is placed at the inlet of a column of suitable granular material and is passed through the column by a carrier-gas. The components eventually leave the column in the gas stream and their concentrations are recorded as a function of the time, or of the volume of carrier-gas, by a detecting instrument. Separation of the components is achieved by repeated equilibration between the mobile gas phase and the stationary phase. Gas–solid equilibria are involved when the stationary phase is an adsorbent, and gas–liquid equilibria when it is a liquid supported on an inert granular medium. Almost any degree of separation can be achieved by varying the nature of the stationary phase and the length and temperature of the column.

In frontal analysis the mixture to be separated is carried continuously on to a column of an adsorbent and at a constant concentration in the carrier-gas. The component which is least strongly adsorbed leaves the column first and each succeeding component leaves the column mixed with a small amount of each of the preceding components.

With displacement development the mixture to be separated is adsorbed upon a column of adsorbent. A constant concentration of a substance which is more strongly adsorbed than any of the components of the mixture is then passed through the column in the carrier-gas. Each component forms a band of constant concentration on the column and emerges from the column substantially pure except for a small overlap fraction between each band.

Although frontal analysis and displacement development formed the basis of G.S.C. prior to the introduction of G.L.C., they have now been largely replaced by elution analysis. They are not used for analytical separations in G.L.C. (although frontal analysis has been used [61, 62] to study the symmetry of peaks) and CLAESSON [101] should be referred to for a detailed treatment of these two methods. The possibility of a form of frontal analysis or displacement development occurring during elution analysis in G.L.C. cannot be neglected, particularly where the sample enters the first section of the column, and where components of a mixture may be present at relatively high concentrations in the stationary phase. The components may then behave non-ideally and tend either to displace one another from solution, or to associate with one another and remain in solution.

Principles governing the elution of substances through G.L. columns

Distinction between column efficiency and solvent efficiency

The overall efficiency of separation of a pair of solutes in a column can be divided into two contributions, (i) the column efficiency, and (ii) the solvent efficiency. The column efficiency is concerned with the spread of an initially compact zone of material as it passes through the column, and can conveniently be described by the height of a theoretical plate (HETP). The HETP is primarily a function of the column design and operating conditions, and to a lesser extent, of the natures of the solute and solvent (*i.e.* of the solvent efficiency). The solvent efficiency, or separation factor, can be expressed as the ratio of the times for two peak maxima to travel through a column, and is characterized by the respective distribution coefficients of the solutes. It is thus a function of the column temperature and of the natures of the solutes and of the solvent.

Theories have been developed to account for the rate of movement, and the characteristic shape, of zones of solutes in chromatographic columns. These theories are classified below and their application to G.L.C. first shown for a single solute. The solvent efficiency will be considered in detail later under principles governing the separation of mixtures of solutes.

Classification of theories of chromatography and their application to G.L.C.

The simplified theories of chromatography can be classified according to (i) the type of distribution isotherm that the solutes obey, and (ii) the ideality or non-ideality of the conditions. With "*linear*" chromatography the distribution coefficient (*i.e.* the amount of solute per unit volume of stationary liquid phase/the amount of solute per unit volume of mobile phase) is independent of the concentration of the solute, and with "*non-linear*" chromatography it is dependent on the concentration of the solute. In "*ideal*" chromatography the flow of the mobile phase is considered uniform, the column is uniformly packed, equilibrium between the two phases is instantaneous, and longitudinal diffusion of solute molecules (or other processes having the same effect) does not occur. In "*non-ideal*" chromatography these assumptions cannot be made.

The four possible types are illustrated schematically in Fig. 1 (after MARTIN [405]).

1. *Linear ideal chromatography* has been dealt with in the classical treatment of WILSON [602]. The shape of the band remains unchanged during elution, mixtures of solutes behave independently, and the requirements for individual bands to be separated can be calculated by simple mathematics.

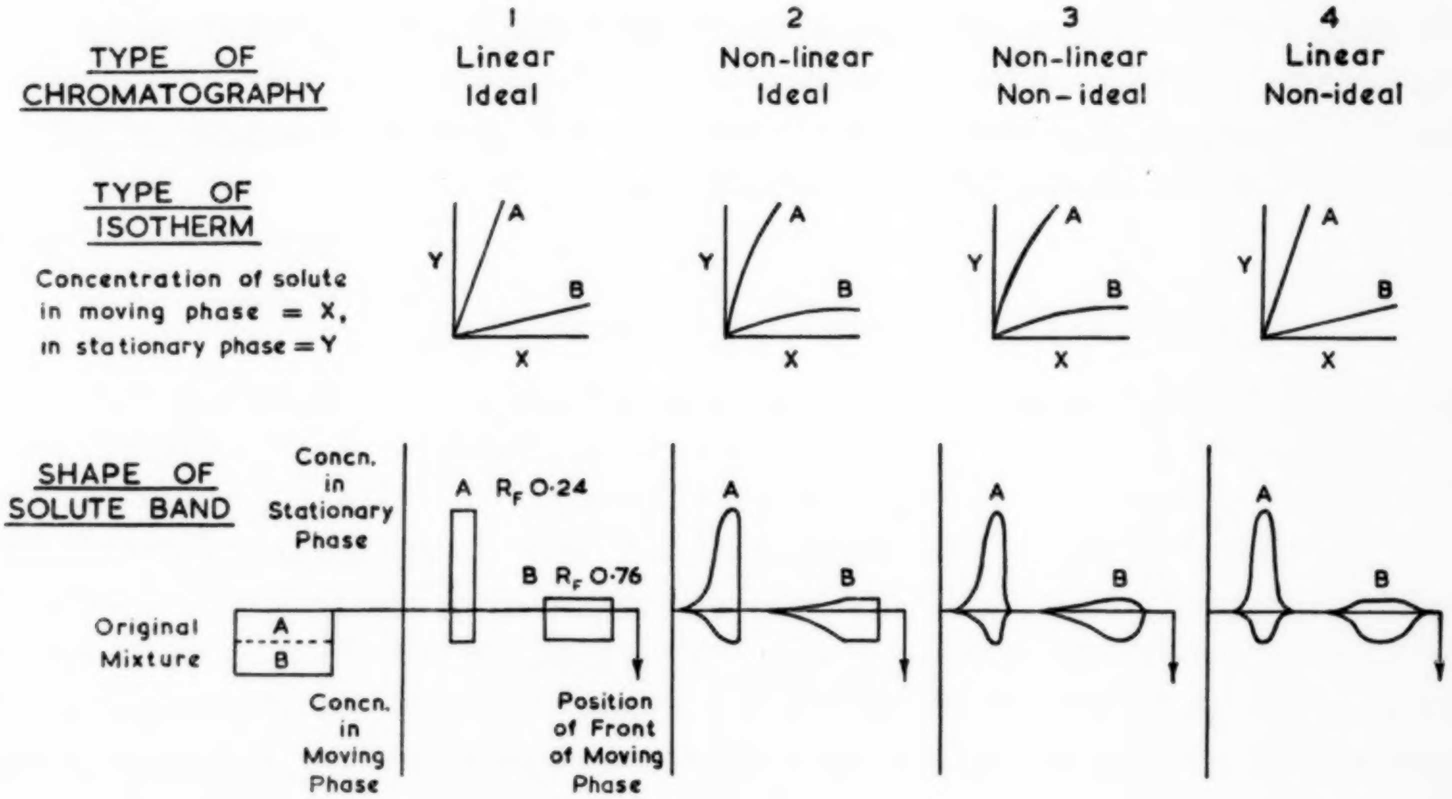
TYPE OF CHROMATOGRAPHY
1 Linear Ideal
2 Non-linear Ideal
3 Non-linear Non-ideal
4 Linear Non-ideal
TYPE OF ISOTHERM
Concentration of solute in moving phase = X, in stationary phase = Y
Y
A
B
X
SHAPE OF SOLUTE BAND
Concn. in Stationary Phase
Original Mixture
A
B
Concn. in Moving Phase
A R_F 0·24
B R_F 0·76
Position of Front of Moving Phase

Fig. 1. Classification of types of chromatography (after MARTIN).

2. *Non-linear ideal chromatography* was first treated by WILSON [602], and later by DE VAULT [150] for a single solute, but solutes can affect the behaviour of each other and rigorous treatment is not possible [353].

3. *Non-linear non-ideal chromatography* has been discussed by KLINKENBERG AND SJENITZER [363], but since G.S.C. is the main type of chromatography concerned and the mathematical treatment becomes very involved, it will not be considered further.

4. *Linear non-ideal chromatography* is of particular importance for the treatment of liquid–liquid and gas–liquid partition chromatography, since the assumption of a linear isotherm is usually a good approximation. The theory has been dealt with in two ways:

(i) By the "plate" theory [217, 412, 415], in which the column is regarded as a discontinuous medium analogous to a distillation column, and in which a finite volume of solution is equilibrated successively with a number of theoretical plates of solvent.

(ii) By the "rate" theory [141, 210, 219, 353, 375], in which the column is regarded as a continuous medium in which mass transfer and diffusion phenomena are taken into account.

The shape of elution curves is given by a binomial distribution for the discontinuous theoretical plate treatment and by a Poisson distribution for the continuous treatment. Although for a sufficiently large number of equilibrations both distributions approximate to Gaussian curves, KLINKENBERG AND SJENITZER [363] have shown that their widths are different. The mechanism of the widening of a band in chromatography has been examined in terms of the "rate" theory by VAN DEEMTER *et al.* [141], who extended the theory developed by GLUECKAUF [219] and other workers. KEULEMANS [353, 357] has recently reviewed the combination of "rate" and "plate" theories, which together give a qualitative and quantitative understanding of the numerous parameters operating in G.L.C.

The three principal contributions to the broadening of a band are:

(i) eddy diffusion due to packing,
(ii) molecular diffusion,
(iii) resistance to mass transfer.

From these a basic equation can be derived [141, 357] for H, the height equivalent to a theoretical plate, in a gas–liquid column:

$$\underbrace{H = 2\lambda d_{\text{p}}}_{\text{eddy diffusion}} \quad + \quad \underbrace{\frac{2\gamma D_{\text{gas}}}{u}}_{\text{molecular diffusion}} \quad + \quad \underbrace{\frac{8}{\pi^2} \cdot \frac{k'}{(1 + k')^2} \frac{d_{\text{f}}^2}{D_{\text{liq.}}} \cdot u}_{\text{resistance to mass transfer}}$$

where

λ and γ = constants,
d_{p} = the particle diameter,
D_{gas} = the gas diffusivity,
u = the linear gas velocity,
k' = $k(F_{\text{liq.}}/F_{\text{gas}})$,
k = the distribution coefficient,
$F_{\text{liq.}}$ and F_{gas} = the volume fractions of liquid and gas in the column,
d_{f} = the liquid film thickness,
$D_{\text{liq.}}$ = the diffusivity in the liquid phase.

This equation is of the form $H = A + B/u + Cu$, where A, B, and C are constants referring to (i), (ii), and (iii) respectively. This is a hyperbola with a minimum H at a certain value of u at which the column is operating most efficiently (see Fig. 2). However, owing to the compressibility of the gas-phase, u is not constant over the length of the column, hence only a small section can operate at maximum efficiency. The influence of the parameters of the equation on the efficiency of separation has been discussed by KEULEMANS [353, 357], and others [140, 141, 394]. The conclusions which have been drawn are of considerable practical interest and are included later under the influence of various factors on the column efficiency.

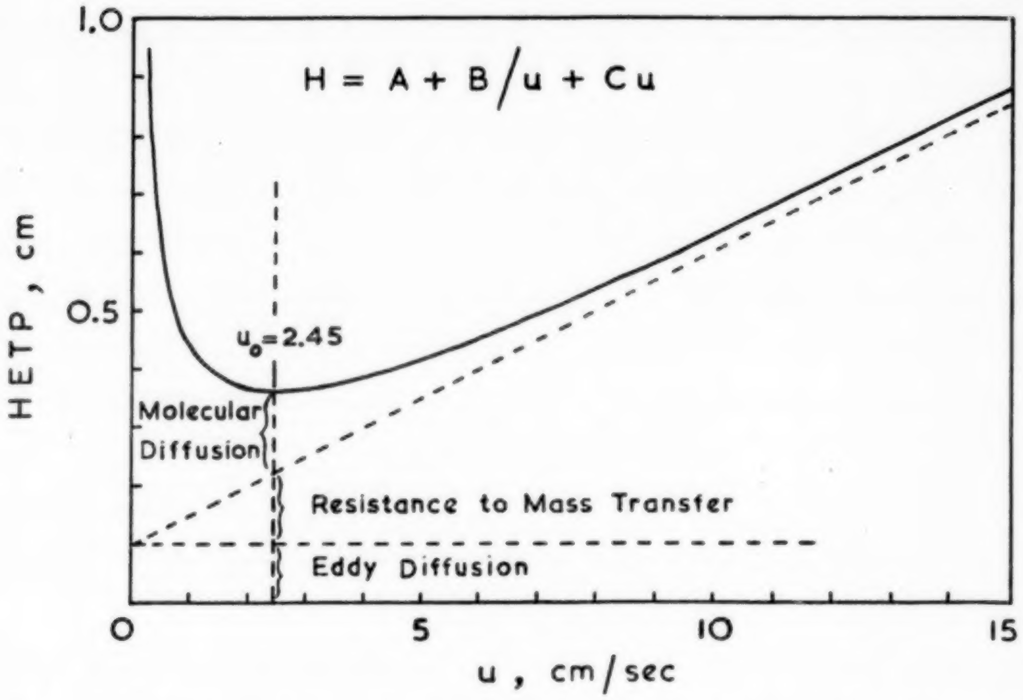

Fig. 2. Plot of HETP against calculated linear gas velocity (after KEULEMANS).

The calculation of the number of theoretical plates

Two methods were originally described [314] for the calculation of the number of theoretical plates in a gas–liquid column. Since then equations for the calculation of plate numbers from recorded chromatograms of elution peaks have been given by a number of workers [64, 395]. These equations give slightly different values and in order to provide a useful practical comparison of column efficiency, the following procedure has been recommended [580]. Tangents are drawn to the elution peak at the points of inflection. The plate number is given by $16(x/y)^2$, where y is the length of the base-line cut by the two tangents, and x is the length from the start of the elution (including the dead-volume of the apparatus) to the middle of the base-line section y.

The effect of various factors on the column efficiency (Table I)

TABLE I

Factor	*Effect on column efficiency (E)*	*References*
Flowrate of carrier-gas	Maximum E for a given value of linear velocity (see Fig. 2).	47, 65, 226, 353, 598, 612
Nature of carrier-gas	E greater for gases of low diffusivity and high molecular weight: $E \propto 1/\sqrt{\text{density}}$.	24, 151
Pressure of carrier-gas	E greater at (i) relatively high pressures and (ii) low ratio of inlet to outlet pressure.	65, 353
Density of packing of column	E constant above a certain density ($\sim$ 0.2 g/ml).	58, 146, 151, 334 353, 357
Particle size of support	Shallow maximum E at $\sim$ 0.1 mm diameter particles.	
Amount of solvent	Optimum at $\sim$ 15–20% w/w for normal columns (see later for very high efficiency columns operated at high inlet pressure).	
Length of column	E increases with length but is not usually directly proportional.	65, 598

(Contd. on p. 7)

TABLE I (*Continued*)

Factor	*Effect on column efficiency (E)*	*References*
Temperature	Complex dependence of HETP on T, *e.g.*: HETP $= A + B(T) + C(T^{-1})$, which is a hyperbola with a minimum H at 24° C.	151
Sample size	E increases as size decreases.	24, 141, 486, 488
Retention volume of solute	E increases with retention volume and the empirical equation holds: log (no. of plates) = const. + ½ log (retention volume).	395, 471, 488
Conditions of sample introduction	(i) Gaseous samples give highest E if introduced in high concentration as a "plug".	353, 492
	(ii) Liquid samples introduced by a syringe give highest E if needle touches column packing; unless this occurs E depends on the temperature of injection.	486
	(iii) Liquid samples introduced in glass bulbs (or by special methods) require careful control to obtain the highest E.	353, 416

Recent developments in the construction of very high efficiency columns

SCOTT AND CHESHIRE [99, 528–530] have recently re-examined the optimum conditions for the construction of very high efficiency columns. They conclude that efficiencies of the order of 900 plates per foot of column can be achieved with narrow columns containing 2.5–5% w/w of liquid phase on close-mesh (100–120 or 120–160) firebrick and operated at high inlet pressure (up to 150 lb./sq.in.) but at a low ratio of inlet to outlet pressure. The latter condition is obtained by placing a very narrow constriction at the end of the column so that the main pressure drop is across the constriction and not across the last section of the column. An example quoted for a 3.6 mm diameter column 9′ 6″ long containing 2.5% of liquid phase on 100–120 mesh firebrick operated at $p_{\text{inlet}}/p_{\text{outlet}} = 2.1$, gave plate numbers from 15,000 to 18,000 for retention times from 30–60 minutes. A mixture which was resolved into 25 components on a "normal" 1500 plate column could be resolved into 50 components on the 15,000 plate column. It is notable that, at the maximum inlet pressure that the apparatus normally withstands, the HETP was still decreasing markedly with increasing flowrate and it is expected that operation at still higher pressure and flowrate should lead to lower values of HETP (*i.e.* < 0.3 mm).

SCOTT predicts that 100,000 plate columns can quite feasibly be constructed based upon the above conclusions and provided that a small sample and a highly sensitive detector are used.

Principles governing the separation of mixtures of substances

The forces of interaction between solute and solvent

In the choice of a stationary phase (solvent) which will give large separation factors between the solutes to be separated, the forces of interaction between solutes and solvent are of great importance. The forces can be divided into four types [359]:

(i) Dispersion, London, or non-polar, forces arising from synchronized variations in the instantaneous dipoles of two interacting species; these are present in all cases, and are the only sources of attraction energy between two non-polar substances.

(ii) Induced dipole, or Debye, forces resulting from the interaction between a permanent dipole in one molecule and the induced dipole in a neighbouring molecule; these forces are usually relatively small.

(iii) Orientation, or Keesom, forces resulting from the interaction between two permanent dipoles, the association energy depending upon both the sizes and positions of the dipoles. (The "hydrogen-bond", *i.e.* the relatively strong interaction between negatively charged atoms and positively charged hydrogen in groups such as OH, NH, and FH, is a particularly important type of orientation force and is of considerable practical use in G.L.C.)

(iv) Specific interaction forces, and chemical bonding, *e.g.* complex formation between solutes and metal ions, or loose adduct formation between solutes and solvents.

These forces of interaction between solutes and solvents govern the relative volatilities of the solutes and therefore the separation achieved. With a non-polar solvent, non-polar solutes will tend to be separated approximately in the order of their boiling points since the forces of interaction (dispersion forces) between solutes and solvent will be similar to the forces between the solute molecules themselves. Polar solutes will be eluted from a non-polar solvent more rapidly than non-polar solutes of a similar boiling point, as they will have lost the strong dipole–dipole association energy which is present in their own liquid [471]. As the polarity of the solvent is increased the polar solutes will be retarded to a greater extent. Solvents of different polarities have been used by a number of workers [63, 316, 359, 395, 570] to effect the separation of mixtures of solutes such as aliphatic, naphthenic, olefinic, and aromatic hydrocarbons. Olefin selectivity increases in a series of solvents of increasing polarity [359], *e.g.* hydrocarbon oil–dinonyl phthalate–dibutyl phthalate–ethyl acetoacetate–dimethylformamide.

By comparing the behaviour of solutes in two or more solvents it is often possible to determine (i) the chemical type and (ii) the position of the solutes in their homologous series. James and Martin [316] examined the behaviour of a series of aliphatic compounds, C_5H_{11}–X, and found that the elution sequence in an aromatic solvent, benzyldiphenyl, is (increasing interaction):

$$X = H < Cl < Br < I < NH_2 < NO_2 < OH < COCH_3 < CN,$$

while in liquid paraffin or a poly-ether the sequence is:

$$X = H < OCH_3 < Br, Cl < I < NO_2 < COCH_3 < NH_2 < CN < OH.$$

The authors stress the usefulness of these sequences, as well as the regular behaviour within homologous series, for the determination of unknown substances.

The retardation of polar solutes can be made very large if H-bonding can take place. A clear illustration of this effect is provided in the early work of James and Martin [313] on the separation of primary, secondary, and tertiary amines using as solvents liquid paraffin, and Lubrol MO (which contains a poly-ethylene oxide structure). The Lubrol MO retards primary and secondary amines due to H-bonding, but does not retard tertiary amines. Comparison of the behaviour of mixtures of amines on these two solvents allows the chemical types of the amines to be identified.

Specific interaction forces probably partly account for the strong retardation of aromatic hydrocarbons by aromatic solvents, *e.g.* the 1 : 1 picric acid–fluorene addition compound has been recommended [359, 595–6] as a solvent with very high selectivity towards aromatic solutes. A solution of silver nitrate in glycol or other solvents has been shown [37, 63, 113, 212, 391, 473] to retard unsaturated hydrocarbons which interact specifically with the silver ion to form weak complexes. Metal salts of fatty acids have recently been proposed as solvents with a high selectivity towards amines [473, 475], and amino-acid esters [33]. These open up a new field in which use can be made of the interaction forces between solutes and metal ions or complexes.

Classification of solute and solvent type

Although the existing theories of solutions do not allow the separation of given solutes by a given solvent to be predicted in more than a few simple cases, a qualitative scheme of classification has been put forward [353]. Five classes of substances are defined in order of decreasing cohesive energy, and a solute will be retained more strongly by a solvent as the classes to which they belong are closer together. However, the classification does not explicitly take into account the effects of mutual polarisability, nor does it allow for the effect of forces, such as those of the chemical bond, which may lead to strong retention of particular solutes by certain solvents.

(I) The molecules form a three-dimensional network of hydrogen bonds. Examples are: water, poly-alcohols, amino-alcohols, oxy-acids, polyphenols, di- and tri-carboxylic acids.

(II) The molecules possess active hydrogen atoms as well as electronegative atoms with free pairs of electrons (O,N,F). Examples: alcohols, fatty acids, phenols, primary and secondary amines, oximes, nitro-compounds and nitriles with α-H atoms.

(III) The molecules possess electronegative atoms but no active hydrogen. Examples: ethers, ketones, aldehydes, esters, tertiary amines, nitro-compounds and nitriles without α-H atoms.

(IV) The molecules possess active H-atoms and negligible dipoles only. Examples: $CHCl_3$, CH_2Cl_2, CH_3CHCl_2, $CH_2Cl \cdot CH_2Cl$, etc., aromatic and olefinic hydrocarbons.

(V) Molecules without functional groups, such as saturated hydrocarbons, carbon disulfide, tetrachloromethane, etc.

The effect of temperature on the solvent efficiency

Since the separation of solutes depends upon the distribution of molecules between the gas and liquid phases, corrected retention volumes (V_R^0, the volume of mobile phase required to elute the maximum of a peak and corrected for dead-volume of column, pressure-drop across column [580], and measured at the column temperature) vary with the temperature according to a Clapeyron-Clausius equation. For small variations of temperature

$$\frac{\mathrm{d}\ln V_R^0}{T} = \frac{\Delta H_S}{RT^2} + K$$

where

ΔH_S = the heat of solution of the solute in the solvent,
K = a constant for different solutes in a given solvent.

The heat of solution is negative (*i.e.* heat evolved) and because of the increasing solute–solvent interaction usually increases numerically with the retention volume.

If V_1 and V_2 are the retention volumes of solutes S_1 and S_2 ($V_2 > V_1$), the separation factor (V_2/V_1) will decrease as the temperature is raised since

$$\frac{\mathrm{d}\ln V_2/V_1}{\mathrm{d}\,T} = \frac{\Delta H_2 - \Delta H_1}{RT^2}$$

In a plot of log V against $1/T$ approximately straight lines are obtained (indicating that this type of equation is obeyed) and the lines for V_1, V_2, V_3, etc. tend to be closer together at high temperatures than at low temperatures (hence smaller separation factor). As a general rule therefore, better separation factors are obtained by operating at low temperatures.

The presentation and correlation of G.C. data

Gas-chromatographic data have been presented in many forms, *e.g.* as retention times, or volumes, relative to various arbitrary standard solutes, and as absolute retention times, or volumes. The committee appointed to investigate the presentation of data has recommended [11, 580] that one of the following should be given (for definitions see below):

(i) *The specific retention volume* (V_g) *or the partition coefficient* (k) for at least two temperatures (if only two then as far apart as possible). The value of V_g or k may be given relative to a standard solute, particularly if this gives a straighter relationship between log V_g and $1/T$.

(ii) An equation relating V_g, or k, and temperature, for example

$$\log V_g = \frac{\Delta H_s}{2.3\,RT} + D,$$

or an Antoine equation,

$$\log V_g = \frac{A}{t + B} + C,$$

where

V_g = the retention volume fully corrected for pressure-ratio across the column, dead-volume of apparatus, sample size, and weight of solvent,
k = the weight of solute per ml solvent ÷ weight of solute per ml of gas phase,
ΔH_s = the heat of solution,
T and t = the temperature in °A and °C respectively,
A, B, C and D = constants determined to fit the equations.

Examples of these methods of presentation are given by YOUNG [613] and AMBROSE [10]. The use of punched cards has been described [547, 603] for the storage and reporting of G.C. data, and this will probably become widely used.

The committee has recommended [580] standard solutes and solvents (see p. 20) and further considers that it is important to include in published work the following: the nature of the support, the nature and weight of the solvent, the sample size (if not corrected for), inlet and outlet pressures, the method of flow-measurement, the accuracy of temperature control, the type of detector, and the density of the solvent (if known) since this enables V_g and k to be interconverted.

Several empirical methods have been used for correlating gas-chromatographic data for solutes in homologous series, and for solutes of similar structure, with other physical data. Straight lines are usually obtained for plots of the log retention volume (or relative retention volume) in a given solvent against:

(i) The number of carbon atoms in the molecule [314, 351, 353, 501],
(ii) The boiling point of the pure solute [180, 235],
(ii) The log of the vapour pressure of the pure solute [283, 284, 496],
(iv) The inverse of the solvent temperature [353, 395, 489],
(v) The log of the retention volume (or relative retention volume) in a second solvent [476]; alternatively a non-log plot can be used [305, 319].

Data in the above form can be used for the qualitative analysis of quite complex mixtures (plots iv and v), for the determination of thermodynamic properties, *e.g.* heats of solution (plot iv, see later for details), and for the determination of retention volumes of solutes (a) for which no standards are available (extrapolation of type i plots) or (b) at temperatures other than the measured ones (type iv plots).

HERINGTON in his thermodynamic treatment [281] of G.L.C. as an example of extractive distillation, deduced the fundamental relation for a pair of solutes in a homologous series:

$$\log V_{21} = \log (p_1{}^0/p_2{}^0) + \log (\gamma_{13}{}^0/\gamma_{23}{}^0)$$

where

V_{21} = the retention volume of solute 2 divided by that of solute 1,
p_1^0 and p_2^0 = the vapour pressures of the pure liquids 1 and 2 at the temperature employed,
γ_{13}^0 and γ_{23}^0 = the activity coefficients of solutes 1 and 2 at *infinite dilution* in the binary mixtures 1, 3 and 2, 3 respectively, where component 3 is the solvent.

Examination of the various terms in this equation using experimental data other than those from G.L.C. reveals the theoretical foundation for many of the empirical relations in current use, *e.g.*, plots of types (i)–(v) above.

Activity coefficients (preferably at infinite dilution γ^0) can be relatively easily calculated from gas-chromatographic data (for details see later), and since they are much less dependent on temperature than are retention volumes or partition coefficients, they can conveniently be used for the characterization of solute–solvent systems.

TABLE

CROSS-REFERENCE OF CLASSES OF SOLUTES

The bibliography references to the original papers in which a particular class of solute has been separated

General class of solvent	Examples of solvents	Class of substance separated				
		Saturated, unsaturated, aromatic, etc., hydrocarbons <C10	Hydrocarbons >C10	Halogenated hydrocarbons	Volatile fatty acids and their esters	Alcohols
Hydrocarbons	*n*-hexadecane (C16)	353, 357, 373		505, 513	373	373
	benzyldiphenyl	149, 316, 319		316	316	316
	squalane	160, 179–181, 373, 425, 476, 492, 570, 617			570	476
	liquid paraffin	28, 113, 151, 172, 316, 496, 512, 526–7, 592–4		28, 254, 316, 488, 497, 498, 596	28, 596	316, 596
	n-hexatriacontane	148–9				
	tri-isobutylene	30, 63, 254				
	fluoro-hydrocarbons			187, 505		
Alcohols	glycerol			10, 488–9		85
	polyethylene glycols	2, 13, 57, 58, 73, 605		187	2, 333–4, 596	2, 52, 58, 137, 333, 596
Ethers	Lubrol MO	318				
Esters	dinonyl phthalate	28, 51, 65, 73, 85, 123, 129, 249, 353, 359, 435, 501–2, 596		10, 36, 186–7, 249, 250, 254, 486, 488–9, 511, 519, 576	28, 487, 501, 511, 596	1, 28, 123, 486, 501, 596
	didecyl phthalate	160, 201, 230, 364, 476, 492, 556		160, 258		160, 258, 476, 492
	other alkyl phthalates	280, 326, 359, 374, 390, 455, 496, 518, 544, 556		187, 238, 465, 488		353
	tricresyl phosphate	83, 241, 379, 523, 566		241, 254, 255, 566	379, 395, 418	379, 395, 523
	tritolyl phosphate	10, 259, 395		259	10, 325, 395	10, 259, 395, 469
	fluorene picrate	595–6				
Silicone and other oils and greases	silicone 702 or 703	10, 249, 250, 395, 473		10, 187, 250, 488–9	10, 325, 395	10, 395, 473
	silicone 550, etc.	156, 440, 469, 538, 595–6, 614	596, 614	188, 231, 254, 366, 417, 488	16–19, 304, 313–4, 334, 343, 418, 469	469, 614
	silicone high vac. grease	51, 429	3, 127	187, 429	3, 38, 117–119, 127, 159, 297, 545, 566	118, 119, 159, 351
	Apiezon L	528	3, 527		3, 38	
	Apiezon M or others	99, 158	336	187	158, 351, 378, 590	
Metal salts	$AgNO_3$/glycol, etc.	37, 63, 106, 113, 212, 391, 473	106			
	metal stearates, caproates, etc.	473				473
Miscellaneous	polythene		127		38, 127, 351	
	sugars					
	dimethylformamide	113, 353, 356, 359, 440, 518, 568, 605				
	dimethylsulpholane	113, 182, 201, 277, 353, 364, 558				
	zeolites and alkyl-ammonium bentonites	31, 327, 592–4, 597		327		31
	nitrobenzene	518		202		

It is not important for the presentation of results whether γ^0 or k^0 (the value of k at infinite dilution) is used, since γ^0 can be calculated from p^0 and k^0 and vice versa. However, γ^0 is particularly useful for discussing solute–solvent interaction and for correlating the structures of solutes and solvents with their chromatographic behaviour. PIERROTTI and co-workers [476, 492] have developed a method (PIERROTTI's "Building Block" Method) for correlating activity coefficients with the structures of the solute and the solvent. They have determined activity coefficients prevailing in a large variety of binary mixtures, so chosen that variations within homologous series of both solutes and solvents could be studied systematically. Although much of this work has involved solvents of volatilities too high to be of direct interest for G.L.C., certain regularities in behaviour are applicable to the understanding, and prediction, of the behaviour in practical G.L.C. systems (for example, see the discussion by KEULEMANS [353]).

II

SEPARATED BY A NUMBER OF SOLVENTS

by a particular solvent are given at the intersection of the respective horizontal and vertical columns.

Class of substance separated										
Ketones	Aldehydes	Ethers	Sulphur-containing compounds	Nitro-compounds and organic nitrates, etc.	Amines and amino-acid derivs.	Boron, silicon or deuterium compounds	Essential oils, terpenes, etc.	Pyridine bases	Phenols and derivs.	H_2O
373	373									
316			149		307					
476, 570		570								
316			512, 527		301, 307, 319	346(B)		74, 313		596
			149							
					313			74, 76, 445		445
2, 52, 57, 137, 596		52	137					74, 76		2, 52, 596
					301, 307, 319			313		
64, 85, 123, 429, 486, 501, 596, 613	85, 123, 501	64, 65, 501, 558	546	75, 249, 250, 487				74, 76		1, 123
160, 258, 476	160					604(D)				
			557	490					344–5	
379	379		7, 523		395	346(B)	384			
259	259	259, 469				346(B)				
473, 488		473			473					
	294				319		167, 384	74, 76, 313		
351	159				33, 34		508			
			270, 566						270, 297	
158, 508		95					289, 290, 508		95	
473		473			33, 34, 473, 475					
									329	
		558								
						202(Si)				

Analytical applications of G.L.C.

A very wide range of substances have now been examined by G.L.C. and examples of the solvents used to separate various classes of substances are illustrated in Table II. In principle all components of mixtures which can be separated (and usually qualitatively analysed) can also be quantitatively analysed by the application of methods suitable to the detector employed, and which are reviewed in Section II.

References to the application of G.L.C. to the complete or partial analysis of some complex mixtures are given below.

Organic fluoro-compounds [21, 185–7, 221, 238, 465, 513–5, 519, 541, 553, 559, 563].

Combustion products of internal combustion engines [88, 148, 182, 195, 272, 295].

Essential oils [41–2, 49, 66, 87, 167, 288–291, 300, 387, 446–8, 468, 508, 552, 569, 588, 609].

Components of tars [72, 234–5, 297–8, 344–5] and tar heavy oils [175].
Naphthalene hydrogenation products [544].
Trace analysis at parts per million level [40, 56, 126–7, 272, 589].
Solvents for plastics and lacquers [126, 259, 260, 372, 378, 596].
Determination of steroid side-chains [111].
Monitoring of distillates from fractional distillation columns [221, 438, 487].
Halogen and interhalogen compounds [184].
Flavours in fruit, vegetables, and other foodstuffs [96, 110, 127, 161–4, 189, 262, 292, 300, 333, 380, 437, 506, 539, 540, 551, 588].
Components of tobacco smoke [460, 464, 498, 532, 534].
Products of Fischer-Tropsch reactions [129].
Products of reaction-kinetics studies [84–5, 104, 123, 170–2, 209, 348, 366, 422–5, 490].
Separation of *o*-, *m*- and *p*-nitrotoluenes [451], and *m*- and *p*-xylene [200, 374, 458].
Volatile fatty acids (and their esters) in natural products [16–19, 24, 38, 109, 115, 117–120, 266–9, 274–5, 296, 311–7, 320–2, 343, 351, 385, 386, 392, 418–420, 453–4, 462, 485, 498, 536–7, 572, 581, 590].
Polymer degradation products [135] and impurities [126, 560, 587, 606].
Insecticides and antioxidants [3, 332].
Radiolysis products of organic compounds [83, 152–3, 165, 188, 195, 230, 507, 600].

The determination of activity coefficients and heats and entropies of solution by G.L.C.

A number of recent papers [12, 14, 284, 353, 373, 395, 492, 593] have shown the usefulness of G.L.C. in the study of the thermodynamics of systems involving volatile solutes and involatile solvents. Activity coefficients and free energies of solution are derived from retention volumes at a given temperature; heats of solution are derived from the temperature dependence of the retention volume; and entropies of solution can be derived from the combination of heats and free energies of solution. KEULEMANS [353] reviewed the methods for the calculation of γ^0, the activity coefficient at infinite dilution, from symmetrical and asymmetrical elution curves and tabulated values of γ^0 for a number of hydrocarbons and oxygenated compounds. However, the data were not regarded as highly accurate since they were derived from incidental analyses carried out for other purposes. A greater accuracy can be achieved in specially designed experiments, for example, the work of KWANTES AND RIJNDERS [373]. These authors also measured values of γ^0 for polar solutes in non-polar solvents by using fine metal helices as the inert support, and extended the method to solvents of relatively high volatility by presaturating the carrier-gas with solvent vapour. In this way, the value of γ^0 for a normal hydrocarbon in its next higher homologue was determined with fair accuracy.

The determination of the activity coefficient at infinite dilution, γ^0

The value of γ^0 is usually obtained in the form of $\gamma_p{}^0$ or $\gamma_f{}^0$ from the equations:

$$\gamma_p{}^0 = \frac{N_{\mathrm{liq.}}\,RT}{k.p^0} \quad \text{or} \quad \gamma_f{}^0 = \frac{N_{\mathrm{liq.}}\,RT}{k.f^0}$$

where

p^0 and f^0 = the vapour pressure and fugacity respectively of the solute at temperature T°A,
$N_{\mathrm{liq.}}$ = the number of moles of solvent per unit volume at temperature T,
k = the partition coefficient derived from:

$$V_R^0 = V_{\mathrm{gas}} + k\,V_{\mathrm{liq.}}$$

where

V_R^0 = the retention volume corrected to the column temperature and average column pressure,
V_{gas} and $V_{\mathrm{liq.}}$ = the volumetric gas and liquid hold-up of the column respectively at the temperature T.

The requirements for the accurate evaluation of γ^0 are:

(i) A very small sample size, or extrapolation of V_R^0 to zero size from a range of small samples.

(ii) Accurate measurement of retention time, dead-volume of apparatus, temperature and pressure of flowmeter, inlet and outlet pressures at the column, and column temperature.

(iii) Relatively long columns (3–6 m recommended [353] to reduce the effects of non-ideality in the first part of the column).

TABLE III

VALUES OF γ_p^0 FOR HALOGENATED HYDROCARBONS

Solute	*Solvent*						
	Silicone 702				*Dinonyl phthalate*		
	*20.2 °C**	*40.1 °C*	*77.0 °C*	*97.9 °C*	*39.7 °C*	*76.8 °C*	*97.9 °C*
CH_3Cl	0.36 (23.1)	—	0.37	—	0.45 (23.6)	0.45	—
CH_2Cl_2	0.38	0.40	0.44	0.50	0.42	0.46	0.49
$CHCl_3$	0.32	0.37	0.40	0.45	0.33	0.38	0.37
CCl_4	0.45	0.50	0.50	0.58	0.71	0.71	0.71
CH_2ClBr	0.42	0.45	0.47	0.51	—	0.49	0.48
CH_2Br_2	—	0.52	0.59	0.61	—	0.60	0.61
$CHBr_3$	—	—	0.65	—	—	—	0.64
CF_2Cl_2	0.75 (21.9)	0.76	0.63	—	0.91 (24.5)	0.80	—
$CFCl_3$	0.56 (21.9)	0.53	0.52	—	0.80 (24.5)	0.66	—
$CHFCl_2$	0.36 (23.1)	—	0.38	—	0.28 (23.6)	—	—
CHF_2Cl	0.76 (23.1)	—	0.71	—	0.58 (23.6)	—	—
cis-Dichloro-ethylene	—	—	0.42	0.51	0.38	0.42	0.42
trans-Dichloro-ethylene	—	—	0.46	0.53	0.52	0.58	0.60
Trichloro-ethylene	—	—	—	0.57	0.46	0.55	0.60

* Other temperatures are given in parentheses.

(iv) Accurate determination of the mass of the solvent and the volume of the solvent at each value of T (which requires a knowledge of the coefficient of expansion), and correction or compensation for any loss of solvent due to its volatility.

(v) The molecular weight of the solvent (γ^0 values cannot therefore be accurately evaluated for solvents containing a mixture of substances in unknown proportions, or for polymers for which accurate molecular weights are not known).

Values of γ^0 which have been calculated by these methods are: aliphatic and aromatic hydrocarbon solutes in C_8–C_{10} hydrocarbons [373], C_{16}–C_{36} hydrocarbons [353, 364, 373], 1,2,4-trichlorobenzene [373], diisodecyl phthalate [353, 364], and polyalkylene glycols [353, 364]; oxygenated solutes in *n*-hexadecane (C_{16}) [373], diisodecyl phthalate [353], and poly-alkylene glycols [353]. Values of γ^0 for several halogenated hydrocarbons in dinonyl phthalate and in silicone oil 702 have been calculated [251] (Table III) from the data of POLLARD AND HARDY [489].

The determination of the heat of solution

The apparent heat of solution (ΔH) and the excess partial heat of solution ($\Delta \overline{H}_S^E$) [492] can be derived from a plot of the logarithm of the partition coefficient (k) against the inverse of the absolute temperature:

$$\frac{\mathrm{d}\ln k}{\mathrm{d}\left(\frac{1}{T}\right)} = -\frac{\Delta H}{R} = -\left(\frac{\Delta H_v - RT - \Delta \overline{H}_S^E}{T}\right),$$

where

ΔH_v = the latent heat of vaporisation of the solute at its boiling point,
R = the gas constant.

Alternatively ΔH can be obtained by plotting $\log_{10} V_g$ against $1/T$ when [395]

$$\Delta H = -2.3R\,(\text{gradient}) + RT^2\,\mathrm{d}\log \varrho/\mathrm{d}T,$$

where

ϱ = the density of the solvent.

Straight line plots are usually obtained over the range 20–100 °C, but when the plots are slightly curved the value of the gradient is taken at the boiling point of the solute.

Heats of solution have been obtained for (i) several hydrocarbons and oxygenated compounds in the solvents squalane and diisodecyl phthalate [492], silicone 702 and tritolyl phosphate [395], (ii) for halogenated hydrocarbons in silicone 702 and dinonyl phthalate [489], (iii) for benzene and cyclohexane in liquid paraffin [14], and (iv) for several hydrocarbons in paraffin wax, liquid paraffin, and dimethyl-dioctadecyl-ammonium bentonite [593].

The determination of the entropy of solution

The entropy of solution (ΔS) of a solute in a solvent at temperature (T°A) can be calculated from the heat of solution (ΔH) and the free-energy of solution (ΔG) by the relation

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

The value of ΔG has been calculated from the retention volume or partition coefficient at a given temperature by the equation [14]

$$\Delta G = - RT \ln k_B$$

in which k_B is Bunsen's partition coefficient defined by $k_B = 273k/T$.

WHITE AND COWAN [593] obtained entropies of solution analogously, but using the chemical potential of the solute ($\Delta\mu_n$) at a definite mole fraction, *e.g.* $n = 0.1\ M$, calculated from the vapour pressure of the solute exerted above a 0.1 M solution under ideal conditions.

Values of the entropy of solution have been calculated from gas-chromatographic data for only a few systems: (i) for benzene — 13.2 e.u., and cyclohexane — 9.5 e.u. in polyethylene glycol cresyl ether [14], (ii) for homologous series in silicone 702 and in tritolyl phosphate [471]; in silicone 702 the addition of 3 carbon atoms along a series resulted in an average change of 1 kcal/mole in ΔG at 70° C, and 3 entropy units in ΔS; in tritolyl phosphate an average change of 2 kcal/mole in ΔG and 6 e.u. in ΔS respectively were found, and (iii) for benzene, toluene, *o*-xylene, hexane, cyclohexane, and heptane in liquid paraffin [593]; values of $-\Delta\mu_n$ and $-\Delta S_n$ from 1.44–1.80 kcal/mole and 15.1–16.9 cal/mole deg. respectively were obtained (n referring to a 0.1 M fraction).

SECTION II. APPARATUS AND TECHNIQUES FOR G.L.C.

This section deals with apparatus for analytical and preparative gas–liquid chromatography and the techniques for quantitative analysis. For elution analysis one requires a supply of a suitable carrier-gas at a constant flowrate, a sample-introduction system, a column containing a stationary phase, and a detector.

Apparatus

The mobile phase

The gases most frequently used for the mobile phase are nitrogen, hydrogen, helium, and carbon dioxide. High molecular weight gases lead to better separation in the gas–liquid column because of the reduced axial diffusion of the solutes, but the choice of mobile phase is also intimately connected with the detector. Hydrogen and helium have lower densities and higher thermal conductivities than nitrogen and both increase the sensitivity of detecting systems using these properties and also simplify the quantitative interpretation of chromatograms. However the efficiency of separation has been shown to be lower for hydrogen than for nitrogen [353, 357]. Carbon dioxide has been used with considerable success [113, 326–31, 353, 502] but the detection method (see later) limits the solutes which can be separated to those which do not condense at room temperature and which are not attacked by alkali.

The column efficiency depends upon the linear gas velocity which is not uniform along the column. There is an optimum velocity for a given set of operating conditions, but usually the gas flowrate is not a highly critical parameter. The volumetric gas rate used depends upon the column diameter, among other factors, and flowrates for 6 mm diameter columns range from about 10–400 ml/min. A value of about 100 ml/min is usually satisfactory for this diameter of column, but for columns of greater diameter the flowrate should be increased in proportion to the square of the diameter, in order to maintain approximately the same linear rate.

The flowrate can be controlled with either a simple manometer [314, 323] or a well constructed needle valve, and measured by a number of methods, *e.g.* capillary, U-tube, and moving-bubble flowmeters, and rotameters. The latter are useful for indicating the flowrate but are not recommended [353, 580] for accurate measurements in the calculation of retention volumes.

The required flowrate is obtained by applying a pressure difference between the column inlet and outlet. Frequently either the inlet or outlet are at atmospheric pressure, but sometimes both pressures are controlled. Very long columns can be used for difficult separations without too excessive a pressure drop across the column provided that they are packed with a coarse supporting material (30–60 BSS mesh). Operation of columns at reduced pressure does not appear to offer any very great advantages [353], and usually leads to less efficient separation. Some gain in detector sensitivity is to be expected from operation of certain detectors at low outlet pressure but thermal conductivity detectors in particular become more pressure-sensitive at low pressure.

The sample and sample-introduction systems

The sample to be analysed may be introduced into the apparatus as a gas, a liquid, or a solid dissolved in a suitable solvent. Volumes introduced can vary from 0.1 ml to 1 μl for liquids and up to 20 ml N.T.P. for gases on a 4–6 mm diameter analytical column. Introduction of liquids with a micrometer syringe and hypodermic needle through a self-sealing rubber cap has become a standard technique, and many improvements of Ray's original method [501–2] have been suggested [91, 119, 571]. The introduction of reproducible amounts of liquids in glass capillaries through large bore metal valves is used in some recent commercial equipment [566–7]. For good resolution the whole of the sample should be introduced as rapidly as possible. This is best achieved by injection of the sample directly on to the column packing or on a glass-wool plug maintained at the column temperature. Alternatively a separate heater can be used at the inlet point to provide rapid vaporization [63, 187, 250, 486, 566] and the effect of the temperature of the heater on the column efficiency and retention volume has been studied [250, 254, 486]. Special methods for the introduction of samples from biological materials are required, *e.g.* liberation of free fatty acids and bases from their salts [54, 314, 418].

Gas samples can be injected directly with a hypodermic syringe [502] but by-pass gas pipettes or mercury burettes are more generally used [248, 256, 353, 568]. By-pass systems are particularly useful for periodic analysis of gas-streams [26, 276, 616] and can be adapted to automatic control. Greaseless systems are of particular importance since the majority of gases and vapours are soluble in greases and can cause severe cross-contamination, especially if trace components are to be analysed. Special lubricants for taps have been used [113, 238] to overcome this problem. Many techniques used for the introduction of samples into mass spectrometers or vacuum systems can be applied to gas chromatography systems [6, 133, 433, 521].

The chromatographic column

Various materials have been used to contain the stationary phase, *e.g.* glass, copper, polythene, and stainless steel, and in many different shapes, *e.g.* straight, coiled, and U-shaped. Typical analytical columns are of the order of 4–6 mm internal diameter and 3–10 foot long. For longer columns, coiling can lead to a considerable reduction in the size of thermostat and the slight loss of efficiency can be tolerated. Columns of larger diameter have been used for the routine separation of larger samples, *e.g.* 3 cm diameter for up to 10 g samples [186, 361].

The column is usually maintained at a constant temperature by a vapour-jacket or a thermostatted oil- or air-bath. Accurate control of temperature is necessary for good reproducibility of retention volumes and accurate quantitative analysis. Vapour jackets are very convenient but the temperature is dependent upon the atmospheric pressure and for long term constancy a thermostat is better. In order to obtain a range of temperature with only one vapour jacket, the pressure above the liquid can be controlled with a manostat, but variable temperature air-baths are becoming widely used [23, 353].

Details have been given of columns operated under "programmed" heating, *i.e.* with the temperature increasing with time [124, 171, 231, 240–1, 246, 255, 523]. This method has advantages for the rapid separation of mixtures of substances having a wide range of boiling points, but a major difficulty is the maintenance of a constant base-line in the detector. Multiple columns in series with take-off points for the slower moving substances have also been used to decrease the analysis time [70, 103, 134, 201, 238, 255, 391, 432, 455, 538, 568, 617].

The stationary phase

(*i*) *The solid support.* Carefully prepared kieselguhr (Celite) has been used by many workers, and details of its preparation and purification have been given [314, 471]. More recently, a coarser form of kieselguhr, ground furnace-brick, has been recommended and the optimum particle size found to be 30–80 BSS mesh [141, 353, 357]. The advantages of the latter are that it is free-flowing and easy to pack into columns, and it has a low pressure-resistance so that long columns can be used without an excessive pressure-drop. The kieselguhr is often not completely inert and its weak adsorption leads to elution peaks with slight tails. This has been partly prevented for fatty acids by treatment with a solution of dilute phosphoric acid [314] or for bases by treatment with methanolic sodium hydroxide [319]. The treatment may saturate a small number of very active sites in a similar way to the reduction of tailing on active charcoal by the addition of a small amount of an involatile liquid, *e.g.* squalane [180–1], or the de-activation of clay by treatment with glycerin in adsorption chromatography

[128]. The latter experiments with glycerinated clay may have been the first G.L.C. separations, although it was not realized at the time.

The method of mixing the liquid phase and the support can influence the separation efficiency of the column and the recommended method is to dissolve the liquid phase in a volatile solvent, to slurry the mixture with the support, and to evaporate the solvent with continual stirring.

Many other types of support have been used with varying success. Silicone rubber vulcanized and ground to a suitable size has been used for the separation of fluorocarbons [186] and can be used either alone as a dry column packing, or mixed with a conventional liquid phase. Glass powder has been used successfully by several workers [123, 394] but samples of some types of glass can give very inefficient separations compared to kieselguhr [488] and the resultant glass–liquid mixture is often difficult to pack. It was concluded [488] that for high-efficiency columns a porous support is preferable to a non-porous one. Stainless steel [545] or copper [373] helices give a very low pressure drop [545] and negligible interaction even with highly polar solutes [373], but the efficiency is low. Sodium chloride crystals have been used [118–9] at high temperature in preference to Celite in order to obtain a lower resistance to flow. Zeolites have been used as supports for liquid phases [326] in addition to their use as a solid adsorbent [31, 326, 592–4, 597]. Another, and perhaps novel, support reported is carborundum [557].

Capillary tubes coated internally with an involatile liquid (*without a porous packing*) have recently been shown to give good resolution and rapid separations, but require a very small sample and a very sensitive detector [158, 228–9]. The potentialities of this development are very great and in the future very high efficiencies are to be expected, *e.g.* a 250-ft. 0.010 in. internal diameter copper capillary coated with approximately 60 mg of squalane separated a 2 μg sample of 9-isomeric C7-paraffins in 50 minutes with an efficiency of 106,000 theoretical plates [143].

(*ii*) *The liquid component of the column packing.* Ideal requirements for the liquid phase are that it should be relatively involatile and stable at the temperature of operation, it should have a low molecular weight and low viscosity, and that it should be readily available in high purity. As many of these requirements are directly opposed to each other, some compromise is effected in practice. Although the selection of a suitable liquid phase for a given separation has often been empirical, considerable attention is now being given to this problem (see p. 7).

The following liquid phases have been recommended [580] for the intercomparison of solutes and for the presentation of retention volumes of new substances (useful temperature range given in brackets):

n-hexadecane (20–50° C)
squalane (20–150° C)
benzyldiphenyl (80–150° C)
dinonyl phthalate (20–100° C)
dimethylformamide (— 20– + 20° C)
diglycerol (20–100° C)

As a standard, a mixture of 20 parts by weight of liquid phase to 80 parts by weight of Celite 545 support should be used [580]. The above liquids are now being prepared in high purity commercially for special use in gas chromatography.

References p. 32/43.

When liquid phases are operated at high temperature, *e.g.* > 100° C for many liquids, a number of problems are encountered including the loss of liquid phase by vaporization and by decomposition [3, 23, 90, 93, 119, 190, 246, 270, 351, 566–7]. The development of stable liquid phases of low volatility is of great importance because of the opportunity of extending gas chromatography to the separation of organic and inorganic materials of low volatility. A number of stationary phases have already been used successfully for long periods at up to 300° C and these include silicone greases, apiezon greases, silicone elastomers, silicone-gum, and polythene. Many other materials have been examined and found to be less satisfactory; for examples, refer to those rejected by KEPPLER and co-workers [351].

The detector

A large number of types of vapour detector have been used for the detection and recording of the separations achieved by the chromatographic column. The detectors can be divided into two main divisions, (A) integral detectors and (B) differential detectors. An integral detector measures some function of the total quantity of vapour which has passed through it, while a differential detector measures some function of the vapour concentration. The latter can in principle be converted into an integral detector by an electronic or mechanical integrator.

The ideal requirements for a detector are: high sensitivity to the presence of a component in the carrier-gas; rapid response; linear response; independence of operating variables such as pressure and flowrate of gas; good base-line stability; simplicity of construction and auxiliary equipment; robustness; low-cost. BOER [53] compared eight types of differential detector on the basis of many of the above requirements and concluded that thermal conductivity detection and β-ray ionization detection appeared to be, at that time, the two systems most suitable for a wide range of applications in routine laboratories. However, the detectors in widespread use are (a) thermal conductivity, (b) gas-density balance, (c) hydrogen flame, and (d) direct measurement of gas-volume after absorption of the carrier-gas. Methods not considered by BOER, or which have appeared since then, are based on interferometry, measurement of dielectric constant, velocity of sound, or ionization potential at low pressure.

A. Integral detectors

(*a*) *Measurement of volume or pressure.* JANAK and co-workers [330] used carbon dioxide as the mobile-gas to carry the separated components of mixtures from the column into a nitrometer containing a concentrated solution of potassium hydroxide. The carbon dioxide was absorbed and the residual gases measured in a burette. The method is limited to gases and vapours which do not condense at room temperature and which are not attacked by alkali. Much valuable work has been done with this simple type of apparatus and automatic recording models have recently been developed [327, 377, 535]. VAN DE CRAATS [113] also described recording integral detectors based on the measurement of the volume, or of the pressure in a constant volume chamber. A convenient method of purification of CO_2 for this detector is given by KEULEMANS [353].

(*b*) *Direct titration.* JAMES AND MARTIN [313-4] directly titrated volatile fatty acids and bases by passing the gas stream from the column through a titration cell, and gave details of automatic and manually operated micro-burettes. A number of modifications of the original methods have since been described [418]. BLOM [50, 51] has described a simple and inexpensive method based on combustion of the vapours over copper oxide. The CO_2 formed is titrated in a glass apparatus with pyridine as solvent and sodium methylate in pyridine–methanol as titrant. This has been applied to 1–10 mg mixtures of a wide variety of organic substances including nitrogen-, sulphur- and halogen-derivatives.

(*c*) *Other methods.* Mercaptans have been estimated [557] by iodine oxidation in a redox half-cell by passing the vapours through the column with nitrogen and absorbing them in a solution of iodine in potassium iodide/70% alcohol which formed a redox half-element. In a 100 μg mixture each component could be quantitatively determined to 0.03–0.04 μmole. With a small excess of iodine over mercaptan the sensitivity was as high as 10^{-9} mole/mV output. However, tertiary mercaptans cannot be oxidized by this method.

For the analysis of fatty acids BOER has described [54, 55] a coulometric titrator coupled to a recorder, and also an electrolytic conductivity cell. The latter is simple and only requires a thermostatted titration vessel containing dilute sodium hydroxide, a conductivity cell, and a slightly modified electronic recorder. It can be adapted to other detecting systems such as bases with sulphuric acids, or ketones with hydroxylamine chloro-hydrate [44, 54]. LIBERTI has also described [381-6] two methods involving coulometric titration, one for components with an active chemical group, *e.g.* acid or base, and the other for general application.

In the first method the vapours are absorbed in a cell containing a suitable indicator and are titrated with a generating electrode connected to a coulometer in which the current can be controlled by a photo-electric relay.

In the second method oxygen is used as carrier-gas and a small furnace at the exit of the column burns each component to CO_2. The oxygen and CO_2 bubble through barium chloride solution in a cell containing a Pt-wire which acts as an oxygen-electrode and shows the presence of each component. Coulometrically generated hydroxyl-ions bring back the potential to its initial value.

JANAK and co-workers [331] have determined halogens polarographically by eluting them from a silica-gel column and following the increase in the Ti(IV) wave after absorption of the halogens in a Ti(III) solution. NEDOROST [449] has given details of a polarographic cell for continuous gas analysis.

B. Differential detectors

(*a*) *Thermal conductivity.* The measurement of thermal conductivity has been used for many years for the analysis of simple mixtures of gases, and details of the general design and operation of thermal conductivity cells (or katharometers) are given by

DAYNES [138], WEAVER [585], and MINTER AND BURDY [434]. The principle of the detector is that if a constant flow of gas passes over a fine wire heated by a constant electric current the rate of loss of heat by the wire is constant. A change in composition of the gas stream will cause a change in heat loss and thus a change in resistance. In practice two wires and two streams are generally used, one stream from the chromatographic column and one from a source of gas of constant composition. The wires are connected in a Wheatstone bridge circuit and any change of bridge output, due to a change of wire resistance, is amplified and recorded.

Thermal conductivity cells have found widespread use in gas chromatography because of their simplicity, low cost, general applicability and comparatively high sensitivity. They were used in 1936 by EUCKEN AND KNICK [471] to detect gases removed from a column of adsorbent and were also used by many the of early workers in G.S.C. and G.L.C.

A variety of metal-block, and glass, cells with straight and coiled platinum and tungsten wires have been described [8, 57, 59, 60, 63, 75, 101, 117–8, 130, 138, 159, 178, 184, 190, 194, 213, 236, 241, 257, 309, 351, 358, 390, 402, 429, 430, 434, 459, 477, 483, 501, 516, 522, 525, 543, 555, 567, 596, 608]. Optimum operating conditions and sensitivity have been studied by many workers and have recently been discussed in detail by KEULEMANS [353].

The general conclusions from studies of the sensitivity and stability of cells operated with various carrier-gases are that a higher sensitivity can be obtained with hydrogen and helium than with nitrogen, but in most cases this leads to lower stability. The use of hydrogen or helium instead of nitrogen can have definite advantages for quantitative analysis since the thermal conductivity of volatile materials is then less dependent upon the type of molecule and the amount of calibration can be reduced. The sensitivity can be increased for small amounts of impurities by combustion of the vapours to (i) CO_2 [236, 272, 450], (ii) methane [618–9], or (iii) hydrogen [236] which are subsequently detected. The behaviour of the thermal conductivity cell depends considerably upon the temperature of the wire and of the walls of the cell. Incorrect choice of wire temperature can lead to loss of sensitivity and even complete insensitivity to particular substances, *e.g.* non-polar molecules [257]. The inclusion of the katharometer in the column heating chamber is not recommended if maximum sensitivity and resolution are desired. Independent heating is advisable if it is necessary to avoid condensation in the cell.

Thermal conductivity cells for continuous operation at high temperature, *e.g.* up to 350° C, have been developed [23, 38, 117–9, 127, 130, 190, 194, 351, 566–7] and some design implications of high temperature operation have been discussed [130, 351]. The problems are largely those of choosing suitable materials to withstand temperatures of 300° C and upwards, particularly for electrical insulation and gas seals. A comparison of copper, stainless steel, and glass cells [351] showed that metal cells could be used for temperatures up to 250° C, and glass cells up to 300° C. With a 0.67 mm diameter glass cell and a 20 μ diameter wire operated at 8 V, a minimum amount of 0.5 μg of methyl caproate could be detected as a peak eluted from a column in nitrogen. A compact and simple detector (up to 500° C) has been described using ‘ glow plugs'' as elements in a diffusion type cell [192–4].

Thermistors are becoming more widely used in place of metal wires and advantages claimed are low dead-volume, and short response time [27, 40, 78, 124, 131–2, 258, 277, 346, 455, 459, 564–8, 570]. Satisfactory thermistor detectors are now operating in many laboratories and more detailed work is available on stability, sensitivity and optimum operating conditions [40, 112, 583]. DAVIS AND HOWARD [131, 132] described a detector which was stable for long periods and had a low noise level provided that the flowrate of gas, and the temperature of column and detector, were kept constant, and that the whole system was allowed to reach thermal equilibrium before samples were eluted. They found that the magnitude and the direction of the signal varied with the distance of the detector from the exit of the column, and proposed that the heat capacity of the gas was measured by a detector in close proximity to the column exit, whereas the thermal conductivity of the gas was measured by a detector at a distance from the column exit. The development of thermistors to work at high temperature with high stability and sensitivity may be difficult due to the resistance–temperature curve falling sharply with increasing temperature. BISHOP [48] has compared a number of commercial thermistors and has shown that the curve relating sensitivity and operating current passes through a maximum (see also ref. [112]). When operated in this region the thermistor detector has a comparable sensitivity to the "wire" thermal conductivity cell. Care must be taken that the thermistor element is not reduced at high temperatures when hydrogen is used as carrier-gas.

(*b*) *Gas-density balance.* CLAESSON [101] in 1946 measured the density of the gas from a chromatographic column by means of a liquid manometer connected to two 6′-long columns of gas, one containing the effluent gas and the other a standard gas.

MARTIN AND JAMES later developed an instrument based on the same principle but having extremely high sensitivity [411]. The instrument, called a gas-density balance, consists of a metal block bored with a series of tubes which are connected together in a manner analogous to a Wheatstone bridge to compensate pressure differences due to the flow of gas. The pressure difference set up between two of the tubes is then a function of the gas-density only, and the density of the effluent gas from the chromatographic column is compared to the density of the carrier-gas from a "dummy" column. Any difference in density causes a flow of gas in a cross-channel in the metal block. This channel contains a flow-detector consisting of an electrically heated filament close to two connected thermojunctions and a flow of gas alters the temperature of these thermojunctions. The thermoelectric output is amplified and recorded, and for small density differences the recorder deflection is linearly related to the density difference of the two gas streams.

MUNDAY AND PRIMAVESI [444] have constructed an electrical analogue of the system to evaluate the performance, and have compared a copper-block gas-density balance with a balance constructed from copper tubing of the same internal dimensions as the tubes in the solid block. The response curves of the two models were very similar, the solid block model having slightly greater sensitivity and lower background noise. The balances gave a response which was linear with the density difference over the range of practical importance, and the authors discussed the reasons for non-linearity at higher density differences. HAWKES has given details [270] of the construction of a gas-density balance for operation at temperatures up to 300° C, but sensitivity decreases as the temperature is raised, *e.g.* the sensitivity at 56° C is 20% less than at 20° C. An instrument constructed of Monel tubing has been described [184] for the analysis of corrosive and highly reactive gases. Many other applications have been reported [38, 149, 234, 296, 302, 309] and the gas-density balance has been used to determine the molecular weight of solutes to ± 4% [388].

(*c*) *Hydrogen flame detector*. This detector was introduced in 1955 by SCOTT [526] and has since been studied in detail by a number of workers [127, 183, 279, 527, 605]. Hydrogen or a mixture containing hydrogen is generally used as the carrier-gas and is burnt at a small jet at the exit of the column. The temperature of the flame is measured by a thermocouple (for flame temperature contours see PRIMAVESI [495]) and the area of a peak on the recorded chromatogram, corrected for the heat of combustion of the substance, is proportional to the weight of substance present. The detector has the advantages of simplicity, low dead-volume, and low cost, and can be applied to gas chromatography at high temperature. SCOTT used hydrogen and nitrogen–hydrogen mixtures as carrier-gases and found a linear relationship between peak height and weight of substance present, but the sensitivity decreased as the retention volume increased. A single straight-line plot was obtained for peak area against sample weight for a number of hydrocarbons. HARRISON [254] found that pure hydrogen is not a suitable carrier-gas for the elution of methane but that 75:25 or 70:30 hydrogen–nitrogen mixtures are satisfactory. WIRTH [605] has reported the accuracy and reproducibility of quantitative results and compared the response of oxygen, water, and oxygenated and chlorinated compounds with the response of hydrocarbons. He used nitrogen carrier-gas and mixed it with hydrogen (from a dummy column) at the column exit. HENDERSON AND KNOX [279] found that the temperature rise of the thermocouple during the combustion of any band is directly proportional to the rate of liberation of heat, and obtained a linear relationship between the peak area per mole and the molar heat of combustion for twenty-four substances.

GRANT AND VAUGHAN have described [233, 235] a micro-flame, or emissivity, detector which measures the luminosity of the flame rather than its temperature. The hydrogen gas from the column is carburetted with benzene or mixed with coal-gas before burning in the detector. The detector is at least as sensitive as the katharometer and possesses several advantages. It is of simple construction and is particularly suitable for use at high temperatures. Also the response depends largely on the nature of the eluted solutes, hence by comparison of the responses of the emissivity detector with those of a katharometer, a classification scheme can be evolved.

MCWILLIAM AND DEWAR have recently described [427–8] an ionization detector using a hydrogen-flame. This is claimed to have extremely high sensitivity (*e.g.*

$S = 1 \cdot 10^9$ mV.ml/mg [53, 160] with a background of 0.1 mV and compares favourably with other types of ionization detector (see later). In its simplest form, a small platinum loop or brass gauze forms the negative electrode and is supported from 5–10 mm above the jet from which the nitrogen–hydrogen mixture is burnt. A potential of from 0–200 V can be applied between the earthed jet and the negative electrode and the output taken across a high resistor between the jet and earth. A similar instrument has been described by HARLEY *et al.* [252].

(*d*) *Ionization detectors.* The measurement of the ionization current in a gas has been used for analytical purposes by a number of workers. A method based on ionization by β-rays from a radioactive source was developed for gas analysis [491] and later applied to detection in gas chromatography [53, 139]. The advantages of this system are that it is very sensitive; calibration is virtually unnecessary since the differential ionization current may be predicted from the ionization cross-sections of the component molecules; the cell is simple to construct and is adaptable to high temperature; and it is inherently insensitive to changes in flowrate of carrier-gas (N_2 or H_2). Disadvantages are that the auxiliary equipment is more elaborate and costly than, for example, the thermal conductivity method, and precautions are necessary in handling the radioactive source. A sensitive and stable detector has been described using argon as the carrier-gas and a β-radioactive source [398], but to reduce or prevent radioactive handling problems, a number of other methods have been considered. The simplest and most effective is that based on a spark discharge operating at atmospheric pressure [399].

HARLEY AND PRETORIUS [253] introduced a detector in which the potential difference was measured across two electrodes in a high-voltage (900 V) discharge tube through which a part of the carrier-gas passed at a few mm pressure. Less than 10^{-11} mole of a hydrocarbon could be detected and a similar figure has been given by PITKETHLY [479, 480] who recommends modified small neon indicator lamps, or aged iron electrodes from neon lamps.

RYCE AND BRYCE [522, 524] have developed a sensitive low-voltage ionization detector. A small fraction of the helium gas stream from the column passes through a leak into a modified commercial ionization gauge operated at $\sim$ 18 V which is not sufficient to produce ions when helium alone is flowing through. Solutes from the column have ionization potentials below 18 V and give rise to a plate current which is amplified and recorded. The sensitivity claimed is 200 times that of the thermal conductivity method and compares with that of the high-voltage detector. The detector is insensitive to changes in ambient temperature, pressure, and flowrate of the main gas stream of which only a small fraction ($< 0.5\%$) is used.

(*e*) *Radioactivity detectors.* GLUECKAUF, BARKER AND KITT [220] followed the separation of gases by measurement of radioactivity and this is the most sensitive method if the materials to be detected are radioactive. EVANS AND WILLARD [188] have recently demonstrated the detection of amounts of the order of 10^{-15} g of $CH_3{}^{80}Br$ or 10^{-13}g of $CH_3{}^{82}Br$, the sensitivity being inversely proportional to the half-life of the respective radioactive isotope, ^{80}Br (18 min), ^{82}Br (36 h). These authors used a Geiger counter, or a sodium iodide scintillation counter coupled to a photomultiplier, amplifier, ratemeter, and recorder, and separated substances from columns operated at up to 200° C. These results indicate that gas chromatography is as effective with materials at "trace"-levels as at macro-levels. KOKES and co-workers [369] also describe the use of a Geiger counter to detect radioactive components separated from catalytic reactor mixtures. Apparatus for the detection of tritium and ^{14}C-labelled compounds by their weak β-radioactivity has been described [4, 79, 231, 400, 441, 507, 610].

(*f*) *Surface potential detector.* GRIFFITHS, JAMES AND PHILLIPS [241–2] introduced a detector based on the measurement of the e.m.f. set up over a vibrating condenser formed by two metal plates, one of which is coated with a suitable surface film (*e.g.* stearic acid). The detector gives a high sensitivity with polar vapours and the sensitivity increases with molecular weight, but it has a slow and non-linear response. However, it has been successfylly applied to displacement analysis in G.S.C.

(*g*) *Other detectors.* MARTIN AND SMART [404], LIBERTI and co-workers [389], and others [200, 272, 531] have applied infra-red analysers to gas chromatography. Organic vapours are passed in the carrier-gas over heated copper oxide and converted to carbon dioxide. With an infra-red analyser sensitive to carbon dioxide a high sensitivity (proportional to the number of carbon atoms in the molecule) is obtained, provided that combustion is complete. The method is then limited to carbon compounds and a considerable disadvantage is the destruction of the chromatographed material. MARTIN AND SMART suggested that for infra-red absorbing vapours a detector sensitive to all the vapours present would suffice but then the sensitivity would not be high, and would vary with the vapour. However, LIBERTI [389] has reported that hydrocarbons can be analysed satisfactorily without combustion by measuring the absorption at the C-H band frequency, 3330 cm^{-1}.

GRIFFITHS and co-workers [241] investigated methods of detection based upon the measurement of (i) the specific heat of the effluent-gas, (ii) the latent heat of adsorption of vapours, (iii) changes in the dielectric constant of a section of the column as the vapours passed through, and (iv) the flow impedance of the carrier-gas. Only (ii) and (iii) have received any recent application. A simple and robust detector of changes in dielectric constant has been designed [578] for use with routine preparative columns, but has high enough sensitivity for many analytical applications.

DUDENBOSTEL AND PRIESTLEY have reported [173] a detector based on the measurement, by thermocouples, of the differential heats of adsorption and vaporization as the carrier-gas passes through a small bed of an adsorbent at the end of the chromatographic column. The detector is sensitive to low concentrations of hydrocarbons in air and was applied in a continuous process-plant to the analysis of propane in propylene. PRIESTLEY reported [580, p. 165] an improvement whereby a thermocouple inserted directly into the end of the column gives a signal which is proportional to the amount of a component passing the thermocouple.

The measurement of sound velocity has been suggested [310] as a method of detection but the present difficulty is the large volume of sample required to give a sensitive and reliable response. Gas interferometers have been used [577, 614] in gas–solid chromatography but such instruments have never come into general use.

Additional apparatus

The collection of fractions is important not only for the application, if necessary, of further methods of analysis to resolve complex mixtures, but also for the isolation of substances in high purity, and for the measurement of their physical properties. Details of apparatus and methods are given under preparative gas chromatography. The measurement of physical properties can easily be achieved on very small amounts of substances, *e.g.* by mass-spectrometry, but larger amounts are usually required for infra-red [39, 165, 207, 447] or ultra-violet spectrometry [401]. However, micro-cells requiring only 0.002 ml of liquid are now available with commercial infra-red spectrometers. JAMES AND PHILLIPS [324] have described a simple micro-Schliermacher apparatus for the determination of boiling points to 0.1° C on 0.02 ml of liquid. LITTLEWOOD [393] has developed an effusiometer for the determination of molecular weights of boron and silicon hydrides to 5% on samples of 10–100 μg. LIBERTI and co-workers [388] have measured molecular weights with an accuracy of about 4% by eluting the mixture of an unknown and known substance in two different carrier-gases of very different molecular weight. From the ratios of peak areas in each gas the molecular weight of the unknown can be calculated.

The combination of mass spectrometry and gas chromatography can be of great value. BRADFORD and co-workers [63] described in 1955 an analysis in which a complex petroleum mixture gave sixteen chromatographic peaks with a thermal conductivity detector, each of which was collected and analysed by a mass spectrometer. Thirty-two components were finally identified in the mixture. DREW and co-workers [172, 422–6, 542] applied mass-spectrometry to the positive identification of chromatographic peaks and the analysis of mixtures having identical emergence times (particularly deuterated hydrocarbons). They also used gas chromatography to separate mixtures of hydrocarbons which have almost identical cracking-patterns so that mass-spectrometric analysis alone had not previously been possible. The combination of gas chromatography and mass-spectrometry has been reported by a number of workers [45–6, 80, 137, 169, 172, 206, 222, 224, 230, 286, 348, 380, 510, 532, 550, 551].

Commercially available apparatus

In the last few years a number of gas chromatography apparatus have become available commercially. At the present time a variety of types are marketed by a large number of manufacturers in the U.S.A. and Great Britain. The apparatus is robust and the reproducibility of quantitative analysis and sensitivity in qualitative analysis of trace materials is usually of a high order. Prices in Great Britain are of the order of £ 600–£ 1000. The most recent development is the application of gas chromatography to continuous automatic analysis of, for example, liquid and gaseous process streams in industry [5, 26–7, 77, 173–4, 176, 191, 198–9, 271, 276–8, 368, 461, 483, 549–51, 591, 616].

Techniques

Quantitative analysis

A quantitative analysis can be obtained from measurements on the recorded chromatograms, the method depending upon the type of detector used and the nature of its response to the components passing through in the carrier-gas. In quantitative analysis with integral detectors the step heights recorded in the chromatogram directly measure the amount of substance separated, for example, in terms of volume, or pressure, of a gas, or μg equivalents of acid or base etc.

With differential detectors in which the chromatogram is a series of peaks on a chart the quantity of a substance can be related to the height of a peak or the area under a peak. The area can be measured by a mechanical or electronic integrator [125, 353, 554], a planimeter [177, 390, 489], cutting out and weighing the chart [159, 516], or by multiplying peak heights by half-widths [116]. With a small sample, the peak height is proportional to the quantity of a component [501–2] and this affords a very simple and rapid method of quantitative analysis. For detectors which have a response dependent upon the nature of the substance in the carrier-gas, calibration for each substance is usually necessary. With thermal conductivity detectors using nitrogen as carrier-gas, calibration is necessary, but with hydrogen or helium carrier-gas (which have thermal conductivities higher and very different from those of the vapours) the response is less dependent upon the type of vapour molecule. However, for the most accurate results, calibration for each substance is recommended [78, 114, 160].

In gas chromatography it is difficult to introduce quantitatively small samples of liquids and gases and to keep the operating conditions constant. These problems can be largely overcome by using either an internal standard or internal normalization.

In the *internal standard method*, a known amount of a suitable substance is added to the sample to be analysed and the peak-areas or -heights are referred to the area or height of the internal standard peak [63, 117–9, 501]. DIMBAT and co-workers [160] investigated the effect of operating variables using a reproducible by-pass sample injection system and found that the height of recorded peaks is sensitive to column temperature but not to flowrate, whereas the peak area is sensitive to flowrate but not to column temperature. They consider that control of column temperature to at least 1° C and flowrate to ± 1% is required for quantitative analysis using peak areas. RAY [501–2] found that 1° C change in column temperature gave 2.4% error in determination of methylcyclohexane by the height relative to *n*-pentane. A 1° C change in column temperature gave only a 0.3% error for the above materials when peak areas were used [160]. The introduction of liquid samples under constant conditions is stressed by POLLARD AND HARDY [486]. They showed that the rate of change of peak height of an internal standard with temperature of injection may be significantly different from the rate of change of height of the peaks being analysed.

In the *internal normalization method* the areas of all the peaks present are added to give a total area which is normalized to 100%. The ratios of the individual areas to this total give the weight percentage amounts directly. Calibration, or correction, factors can be applied in this and the previous method when mixtures to be analysed contain substances whose responses to the detector are not independent of the type of substance, *e.g.* correction for heats of combustion with the hydrogen-flame detector [527], and for thermal conductivities [78, 358, 430, 516, 568].

The accuracy and reproducibility of quantitative analysis varies with many factors such as the type of detector, the method of analysis used, and the control of operating conditions. The simple detectors based on volume measurement or titration are claimed to be highly accurate (± 0.1% for the JANAK technique) but the sensitivity of these methods is somewhat limited, *e.g.* by the difficulty of accurate measurement of very small volumes of gas. The other detectors and methods, while more versatile and sensitive, can seldom be claimed to give results to better than ± 1% of each component, and it may not be possible to achieve this for the analysis of trace-components. However, it may sometimes be desirable to sacrifice some accuracy or reproducibility for speed of analysis. The reproducibility of particular methods of analysis has occasionally been quoted in terms of the standard deviation. DIMBAT and co-workers [160] reported standard deviations of 7–10% for methods using peak areas and a thermal conductivity detector without calibration, and 0.76% with prior calibration. POLLARD AND HARDY [489] give an average standard deviation of 1.4% for three determinations of halogenated hydrocarbons using peak heights relative to an internal standard. The difference between the amount found and the amount taken was usually under 2% for this method. PERCIVAL [465] analysed fluorinated hydrocarbons and gave 95% confidence limits for the mean of duplicates of about ± 0.5% at the 50% level for any component. The accuracy was within the reproducibility limits. LICHTENFELS *et al.* [390] report an average error of 0.6 mole % for the analysis of an 18-component mixture of hydrocarbons by peak areas, but all the peaks were not completely resolved.

Preparative applications of G.L.C.

Gas–liquid chromatography is primarily a batchwise method of separation of mixtures and has been developed more extensively as an analytical technique than as a technique for the preparation of pure substances. Analytical scale columns can be used to isolate constituents of complex mixtures in small amounts suitable for further study, *e.g.* by mass-spectrometry, but for very complex mixtures or small amounts of impurities, larger samples are desirable.

The amount of sample which can be eluted through a gas–liquid column can be increased in proportion to the cross-sectional area of the column without seriously impairing the efficiency of separation. EVANS AND TATLOW [186–7] separated g amounts of mixtures of fluorinated hydrocarbons on columns 16 ft. long by 3 cm diameter and more recently 16 ft. by 7.5 cm diameter [185]. The latter columns are capable of routinely dealing with 10–70 g of volatile mixtures and give separations previously unobtainable by fractional distillation because of azeotrope formation and the close similarity of boiling points. Compounds boiling 1° C apart, 3H- and 4H-nonafluoro-cyclohexene, were separated from each other. WHITHAM [595] has applied medium-scale, 830 cm (27 ft.) × 13 mm diameter, columns to the analysis of up to 3 ml liquid samples of complex petroleum fractions. BRADFORD, HARVEY AND CHALKLEY [63, 256] in 1955 also used comparable diameter columns for separating 0.5 ml samples of liquid hydrocarbons. They overcame any decrease in separation efficiency (due to larger sample size) by eluting at a lower column temperature. ROBB AND VOFSI [511] separated 500 mg quantities of mixtures of vinyl acetate and bromotrichloromethane on 140 cm (4 ft. 8 in.) × 10 mm diameter columns, and obtained quantitative recoveries to 1.5 mg. Several other applications have been described [66, 71, 92, 361, 371, 551, 559, 579].

AMBROSE AND COLLERSON [9] developed an apparatus for preparative gas chromatography in which the cycle of batch operation was automatically repeated. As each substance emerged at a constant time after sample-injection a clock was used to control the cycles, and solenoid valves directed the samples to collecting traps. The authors used 100 cm × 15 mm diameter columns and sample charges of slightly less than 1 g, but the method is applicable to any size of column. The success of the method depends upon the chromatogram remaining in phase with the clock. Constant elution times were achieved by control of column temperature, carrier-gas flowrate, and presaturation of carrier-gas with the stationary liquid phase. Developments of this type of apparatus have recently been described by a number of workers [25, 273, 287, 371].

The development of efficient collecting systems for samples eluted from gas chromatographic columns has received considerable attention, and a number of techniques have been discussed recently (see ref. [580]: *V.P.C. Symp.*, pp. 96, 209). Simple U-tube traps cooled in liquid nitrogen have been used with varying degrees of success. A relatively low pressure at the column outlet is claimed to facilitate quantitative condensation of components. Expendable traps have been developed with low hold-up volume and sintered glass packing for the recovery of very small fractions (less than 30 μg) from reaction kinetics studies [171].

The rapid cooling of mixtures of a carrier-gas and a condensable vapour often leads to loss due to the formation of fog or minute crystals. Without using filters a good recovery can be obtained by placing several cooling traps in series and heating the gas stream between the individual traps sufficiently to evaporate the floating droplets or crystals [580, p. 96]. GLUECKAUF [580, p. 97], finds that if traps are filled with cotton-wool or some other material on which droplets can collect, there is complete condensation up to the vapour pressure of the substance concerned. If the vapour pressure of the substance is too high at liquid nitrogen temperature, then a small plug of active charcoal can be used. Amounts of less than 10^{-12} g of krypton and xenon, were quantitatively retained by this method. AMBROSE [580, p. 210] reported that active charcoal retained organic materials too strongly and recommended activated alumina. An electrostatic precipitator is stated to overcome fog formation completely [25].

Under certain conditions, small 4 mm diameter gas–liquid columns can be considerably overloaded in order to prepare small samples of pure substances, *i.e.* liquid samples of the order of 0.3 ml can be eluted instead of the normal 1–10 μl [487]. The required substance must have a sufficiently different retention volume from the impurities present. If the retention volumes of impurities and the major component are very similar, the column cannot be overloaded to the above extent, and a larger column is required. Pure ethyl nitrate and formic acid have been prepared by this method.

CONCLUSIONS

Gas chromatography has been used to separate complex mixtures of substances in amounts ranging from 10^{-15}–70 g and with boiling points from -200° to $+400^\circ$ C. It can both qualitatively and quantitatively analyse these substances with good accuracy and in the comparatively short time of a few minutes to a few hours. The separated substances can then be recovered in a high state of purity. Not only is it an outstanding technique on its own, but it can also be used in conjunction with methods such as fractional distillation, mass-, and infra-red-spectrometry, and can achieve results which were previously not possible with existing methods. The appearance of commercial instruments designed for application to research, to the routine analysis of all types of substances, and to process-control in industry, is greatly aiding in the adoption of gas chromatography as a universal separation-process.

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NON-EQUILIBRIUM AND DIFFUSION: A COMMON BASIS FOR THEORIES OF CHROMATOGRAPHY*

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INTRODUCTION

There are three theories commonly used to describe the position and structure of bands in chromatography (we limit our discussion to cases involving linear sorption isotherms; *i.e.*, nearly all partition chromatography). These are the theoretical-plate model[1,2], the conservation of material approach[3,4], and the stochastic theory[5,6,7]. The material-conservation approach and the stochastic theory are very closely related. For this reason they have been collectively termed as "rate" theories[8]. With a given set of kinetic parameters describing transitions between phases (mobile and stationary), it is the scope of these theories to predict the structure of the elution curve. The relative advantage of one theory over the other depends upon the ease of application to specific examples.

The theoretical-plate model ("plate" theory) is of a different nature. The parameter (HETP) for this model must be measured in a given experiment. The model then describes the development of a chromatogram in terms of this parameter and the R value**.

These two areas of approach can be compared to the relationship between statistical mechanics and thermodynamics. The former depends on specific information concerning microscopic events in order to derive macroscopic results, while the latter concerns general rules, equally valid for simple and complex underlying microscopic behavior.

This relationship between the rate and plate theories tells us beforehand the limitations of a treatment comparing them. Since no kinetic parameters enter the plate theory, the results of this theory cannot be directly checked against those of the rate theories. However, a comparison of the results yields a relationship between (HETP) and the kinetic parameters. The treatment presented here is more fundamental than this. Instead of starting with the results (elution or band structure) of plate theory, we have gone back to the basic concept that the chromatogram can be divided into discrete cells, and that the length of the cell (HETP) is related to an equilibrium condition between the content of the cell and its effluent. The latter relationship,

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** We will hereafter use R as the equilibrium ratio of the amount of solute in the mobile phase to the total amount of solute. We may also interpret R as (1) the probability that a particular molecule at a given instant is in the mobile phase, or (2) the ratio of the velocity of the center of the band of solute, u, to the average velocity in the mobile phase, v. For one-dimensional flow (*i.e.* not circular chromatography) R may usually be equated to R_F.

since it concerns non-equilibrium kinetics, can be directly compared to the results of the kinetic relaxation-time model[9]. This method not only yields the desired relationship between (HETP) and the kinetic parameters, but illustrates the nature and limitations of the theoretical-plate model as applied to chromatography and related procedures.

The relationship between the rate theories is different, since they can be directly checked against one another. There are two ways to effect this comparison. Following an obvious method, we could stipulate a given set of boundary conditions relating to column input, obtain solutions by the two methods, and directly compare them. This method will be used in later publication. We have found a method that gives far better illustration of chromatography as a non-equilibrium phenomenon. One restriction is necessary, but this is a very practical one. Our treatment is restricted to those cases where the departure from equilibrium is not large. Once this is allowed, the treatment becomes very general in that it does not depend upon a particular set of boundary (input) conditions. This is done simply by relating the structure of the band to an effective diffusion coefficient, given in terms of the kinetic parameters. It is suggested that the spreading of bands due to this process be termed chromatodiffusion following an analogous term of MYSELS[10] (Electrodiffusion).

Other effects are operative in chromatography that cause spreading of component bands[5]. Ordinary molecular diffusion in the longitudinal direction is always occurring, both in the mobile and stationary phases, though the latter may be negligible. The flow of solvent through a porous media always adds an additional diffuseness (the so-called eddy diffusion) to an included solute band[11]. Both of these two effects can be computed in theory just as chromatodiffusion can. It is then necessary to add the individual diffusion coefficients together to obtain the overall coefficient for diffusion in the chromatogram. In working with the theoretical plate model, it has been shown that (HETP) is the sum of three terms, each stemming from a single one of the above sources of diffusion[8]. In the treatment here we will be concerned with chromatodiffusion, and thus the diffusion coefficient D and (HETP) that we discuss will simply be the contribution due to kinetic effects.

Any of the above effects can be important in chromatographic separations, although in those procedures where the flow velocity is unduly increased for rapid completion of the process, the kinetic effects dominate. For gas chromatography, the relative values have been experimentally determined[8, 12].

It would be clearly advantageous, in many cases, to consider chromatography, from the beginning, as a diffusion process. The voluminous literature concerned with the solutions of the diffusion equation can be immediately applied to a multitude of boundary conditions for chromatography.

THE STOCHASTIC THEORY

The stochastic theory of chromatography is concerned with the movement of a single molecule through a chromatographic column. The successive sorptions and desorptions are comparable to random walk processes, and mark a fruitful field for the application of probability theory. It is to be noted that the problem of equilibrium within the chromatogram arises only in a very limited sense from the point of view of the stochastic theory. This results since the theory, for the most part, is concerned only with the time-dependent behavior of single molecules. However, if a molecule is applied to the column in the mobile phase, then the probability that the molecule will be found in the mobile phase is unity at the instant of application, and asymptotically approaches R as time elapses. This probability is related to equilibrium; the relaxation time for the approach of the probability to its asymptotic value is identical to the relaxation time for the approach of a large collection of molecules to their equilibrium values.

The point of real interest in chromatography, however, concerns equilibrium only in a local sense. In any useful chromatogram, the total concentration of solute in the mobile phase divided by the total concentration, for any calculational purpose, equals R. However, at a given point on the chromatogram, local equilibrium does not obtain, and a deficiency of concentration in the mobile phase at one point is balanced by a surplus at another point. We will expand on these concepts in our discussion of the material-conservation approach, since there we will find it necessary to quantitatively evaluate the local non-equilibrium effects.

In a previous publication, chromatography was considered as a diffusion process[13]. Through expanding the probability density function in a Taylor series along the lengthwise coordinate, the diffusion concept was found to be valid whenever the average number of sorptions is large. The validity of the diffusion concepts can be more fundamentally shown by the methods used by EINSTEIN concerning Brownian motion[14]. It is shown in that treatment that diffusion results from a large number of independent, random displacements. The conditions are the same, since each sorption is independent of the previous one, and the large number of them is stipulated.

When the diffusion conditions are fulfilled, the diffusion coefficient for a band on the column, due to kinetic effects (chromatodiffusion), is

$$D = \frac{k_1 k_2 v^2}{(k_1 + k_2)^3} \tag{1}$$

where v is the longitudinal component of flow velocity, and k_1 and k_2 are the average number of sorptions and desorptions, respectively, in unit time.

THE MATERIAL-CONSERVATION APPROACH

Since the following treatment is concerned with general systems involving reaction kinetics, it is necessary to outline chromatography as a kinetic system. It has been shown that the kinetics of chromatography can be reduced to that of simple kinetic analogs. The simplest one, adequate in most cases, is

$$\mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{B} \tag{2}$$

where A represents a molecule in the mobile phase, and B a molecule in the stationary phase. The arrows indicate the continuous transition between the two "configurations". The transition rates, k_1 and k_2, have been defined.

In order to discuss both the material-conservation approach and the theoretical-plate model, it is necessary to use a method for treating the kinetics of chemical systems near equilibrium. The relaxation-time model for chemical kinetics[9] has been devised for this purpose, and is easily adapted to the treatment of chromatographic processes.

If the concentration, c_1, of a chemical species, A, is perturbed slightly from its equilibrium value (more generally, from its quasi-equilibrium value), it will return to the equilibrium in the manner of exponential relaxation. The relaxation time, t_r, for this process can be easily obtained[9].

$$t_r = \frac{c_1 - c_1^*}{-\mathrm{d}c_1/\mathrm{d}t} \tag{3}$$

where c_1^* is the equilibrium concentration of A.

Referring, now, to the kinetic model of this section, A represents a molecule in the mobile phase, and its equilibrium concentration is

$$c_1^* = \frac{k_2 c_2^*}{k_1} \tag{4}$$

where c_2^* is the concentration of B, and the stars denote the equilibrium. All concentrations are referred to a unit volume of the overall chromatogram. For this model, the relaxation time is found to be

$$t_r = 1/(k_1 + k_2) \tag{5}$$

Let us examine a small volume of carrier fluid as it passes along the chromatogram. We will assume that the concentration, c_1, in this element is near the equilibrium value, c_1^*. However, c_1^* varies as the volume element moves along the column. The concentration, c_1, lags behind c_1^*, since equilibration is not instantaneous. It is shown elsewhere[9] that the time lag is just the relaxation time for equilibrium, t_r. This is described by the equation

$$c_1(z,t) = c_1^*(z,t - t_r) \tag{6}$$

where $c_1(z,t)$ is the concentration in the moving volume element at the point z and the time t. In the discussion of the theoretical plate model, it will be shown that the concentration $c_1(z,t)$ may be considered equal to the equilibrium concentration, either at a time t_r past, as above, or at the same time but a distance $vt_r(1 - R)$ upstream.

$$c_1(z,t) = c_1^*(z - vt_r(1 - R),t) \tag{7}$$

From this equation it is necessary to find the equilibrium departure in the mobile phase, defined by $\Delta c_1 = c_1 - c_1^*$. (This is the negative of the equilibrium departure in the stationary phase, since $\Delta c_1 + \Delta c_2 = 0$.)

$$\begin{aligned} \Delta c_1 &= c_1^*(z - vt_r(1 - R),t) - c_1^*(z,t) \\ &= -\frac{\delta c_1}{\delta z} vt_r(1 - R) \end{aligned} \tag{8}$$

Both Δc_1 and $\delta c_1^*/\delta z$, of course, are evaluated at the distance z and time t. The latter relation holds for values of t_r, and hence Δc_1, that are small.

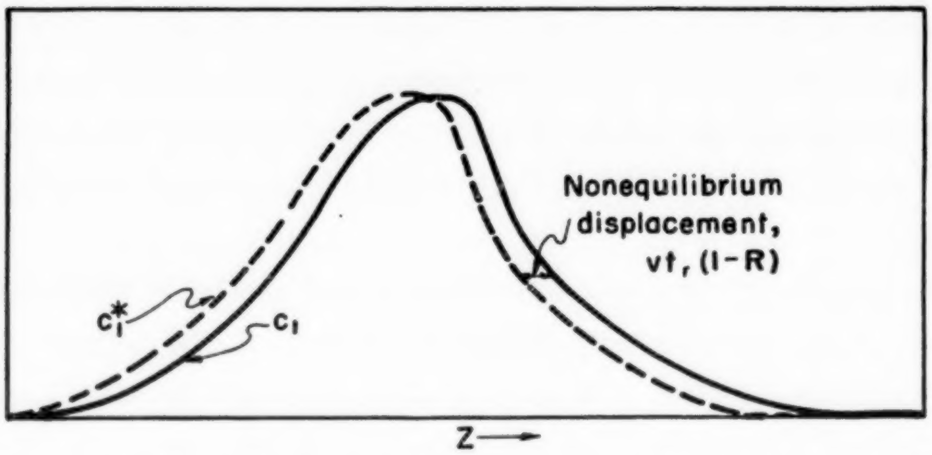

Fig. 1. Schematic diagram showing the shift of the actual mobile phase concentration, c_1, downstream from the equilibrium concentration, c_1^*.

Fig. 1 illustrates the application of this equation to a solute on a chromatographic column. The mobile phase moves in the direction, z, along the column. It is seen that the actual concentration, c_1, is displaced downstream from the equilibrium concentration, c_1^*, a distance, $vt_r(1-R)$, equivalent to a time, t_r. The figure shows how the departure from local equilibrium varies from one point to another on the column.

The mathematical expression of the equilibrium departure is particularly simple when the concentration profile is "Gaussian" or "normal". If we divide each side of equation (8) by c_1^* we get the fractional departure

$$\frac{\Delta c_1}{c_1^*} = -\frac{\delta \ln c_1^*}{\delta z}\, vt_r\, (1 - R) \tag{9}$$

Since c_1^* is a definite fraction of the total concentration, it too is Gaussian.

$$c_1^* = \text{const. } e^{-\frac{(z-\bar{z})^2}{2\sigma^2}} \tag{10}$$

Whenever such a peak results from diffusion, the standard deviation and the diffusion coefficient are related by $\sigma^2 = 2\,Dt$. Using this and taking the derivative $\delta \ln c_1^*/\delta z$ we have

$$\frac{\Delta c_1}{c_1^*} = vt_r \frac{(1 - R)\,(z - \bar{z})}{2Dt} \tag{11}$$

For the A ⇄ B model we may use D from equation (1), t_r from equation (5), and as the fraction of time spent in the mobile phase, $R = k_2/(k_1 + k_2)$. The resulting expression is

$$\frac{\Delta c_1}{c^*_1} = \frac{z - \bar{z}}{2Rvt} \tag{12}$$

the term Rv is the average velocity of the zone and this multiplied by the time t is the displacement of the zones center, $\bar{z}$. The final simple result is

$$\frac{\Delta c_1}{c_1^*} = \frac{z - \bar{z}}{2\bar{z}} \tag{13}$$

This shows the departure from equilibrium to be a function of the distance z along the chromatogram. At the center of the zone it approaches zero. It can also be shown that at a distance σ from the center of the zone, $z - \bar{z} = \sigma$,

$$\frac{\Delta c_1}{c_1^*} = \frac{1}{2\sqrt{N}} \tag{14}$$

where N is the theoretical plate number occupied by the center of the zone. The results given in the last two equations are valid with any kinetics as long as a t_r can be found for the system.

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It can now be easily shown why the spreading (diffusion) of a band is related to non-equilibrium values. At the center of the band, the departure from equilibrium is negligible. This portion of the band is displaced down the column with a velocity, $\bar{u} = Rv$. On the upstream (trailing) side of the band, the concentration is less than equilibrium. This deficiency in the mobile phase causes this part of the band to move more slowly than the center. On the other hand, the surplus concentration on the downstream (leading) side of the band causes this part to move more rapidly than the center. The net result is the spreading of the two edges away from the center.

For a quantitative formulation, we will refer our calculations to a point moving downstream with the average velocity of the band, $\bar{u} = Rv$. The flux of material through a unit area of a plane that is perpendicular to the flow direction and includes this point consists of contributions from all possible phases containing the solute,

$$q = \Sigma\, c_i u_i, \tag{15}$$

where c_i is the concentration in phase i, and u_i is the average velocity of this phase with respect to the reference point. For most cases it is ample to consider just a single mobile phase (phase 1), and a single stationary phase (phase 2). For the velocities in the last equation, we have $u_1 = v - \bar{u} = (1 - R)\, v$, and $u_2 = -\bar{u} = -Rv$. The flux, then, is

$$q = v\,[c_1\,(1 - R) - c_2 R] \tag{16}$$

and since $(c_1 + c_2)$ equals the total concentration, c

$$q = v\,(c_1 - Rc). \tag{17}$$

Using the definition of Δc_1 and the equilibrium expression, $c_1{}^{\star} = Rc$, we have

$$q = v\Delta c_1 \tag{18}$$

At equilibrium, of course, $\Delta c_1 = 0$ and the net flux vanishes.

The value of Δc_1 in this equation for the net flux can be obtained from equation (8). Substitution yields

$$q = -\frac{\delta c_1{}^{\star}}{\delta z}\, v^2 t_r\,(1 - R), \tag{19}$$

and since $c_1{}^{\star} = Rc$, then

$$q = -\frac{\delta c}{\delta z}\, v^2\, t_r R\,(1 - R) = -D\,\frac{\delta c}{\delta z} \tag{20}$$

the latter relation results since D can be so defined in terms of the net flux, q. Hence a very general expression for the effective diffusion coefficient for this process is

$$D = v^2\, t_r R\,(1 - R) \tag{21}$$

This equation can be more specifically written in terms of the kinetic analog, (2). For this particular analog, $t_r = 1/(k_1 + k_2)$, and $R = k_2/(k_1 + k_2)$. With these substitutions, (21) becomes

$$D = \frac{k_1 k_2 v^2}{(k_1 + k_2)^3} \tag{22}$$

a result identical to that derived from the stochastic theory.

An approach similar to the above has been used in the description of boundary spreading in electrophoresis[15]. The mathematics of electrophoretic and chromatographic spreading are the same[13]. In either case an interconversion of species exists with the various species moving at different velocities. The interconversion reaction may be as simple as $A \rightleftarrows B$ or much more complicated for either electrophoresis or chromatography. When this simple reaction occurs in electrophoresis the effective D is the same as in equation (22) except that v_2 must be replaced by $(v_1 - v_2)^2$. The latter are the respective velocities of the ionic species. This result is not the same as that obtained in the work of FIELD AND OGSTON[15].

THE THEORETICAL-PLATE MODEL

The theoretical-plate model serves to relate chromatography to classical distillation procedures. For a given experiment under given conditions, the development of a chromatogram is related to an empirical parameter, (HETP), or the height equivalent to a theoretical plate. With a given column length, L, the number of theoretical plates, N, is simply obtained as $L/(\text{HETP})$.

We will relate the parameters, (HETP) and N, directly to the kinetic parameters involved in the other theories. This, of course, is exceedingly useful, since the theoretical-plate model has had the disadvantage of not being related to fundamental physical quantities, such that, for instance, one could not find an expression for the temperature dependence of N even though it is known for the kinetic rates of the underlying processes.

The theoretical-plate model is obtained by dividing the chromatogram into discrete, adjacent cells. The length of a cell, (HETP), is determined by the condition that the mean concentration in the cell is in equilibrium with its own effluent. For small (HETP), the concentration at the midpoint of the cell is approximately equal to the mean concentration. Thus (HETP) is determined by the condition that the midpoint concentration is in equilibrium with the small volume element leaving the cell. However, the small volume element, according to equation (6), is in equilibrium with the point on the column crossed a time, t_r, previously. Since the flow velocity within the column is v, this equilibrium point is the distance vt_r upstream, and this must be the distance between the end of the cell and its midpoint

$$vt_r = \frac{(\text{HETP})}{2} \tag{23}$$

Thus we have

$$(\text{HETP}) = 2vt_r, \qquad N = t_1/2t_r \tag{24}$$

Where L/v is t_1, the passage time of the carrier through the chromatogram. This is the most general expression of the theoretical plate parameters in terms of kinetic processes. For the $A \rightleftarrows B$ kinetic model, equation (2), we have (see equation (5))

$$(\text{HETP}) = 2v/(k_1 + k_2), \qquad N = (k_1 + k_2)\, t_1/2 \tag{25}$$

These equations can be easily verified. This is done simply by comparing the band half-width obtained by MAYER AND TOMPKINS[2], expressed in terms of N, to the half-width obtained in the use of the stochastic theory. For the $A \rightleftarrows B$ model, the result is identical to (25).

It has been tacitly assumed in the foregoing treatment that the volume element leaving a cell is in equilibrium with the midpoint cell concentration, not as it has been modified by incoming fluid, but as if it had remained constant as the volume element moved through the cell. Thus we have treated the MAYER AND TOMPKINS[2] discontinuous flow model of chromatography. The continuous-flow model, introduced by MARTIN AND SYNGE[1] in the original theoretical plate treatment of chromatography, is a better physical model. Our method for relating N to the kinetic rates involves, in this case, the additional concept of concentration displacement.

For a small downstream displacement of the mobile phase, say a distance vt_r, a given segment of the overall concentration profile will move downstream a distance $c_1vt_r/(c_1 + c_2)$. Since the concentrations c_1 and c_2 are near their equilibrium values, the concentration displacement is approximately Rvt_r. The relaxation-time model states that the concentration of a given volume element is the equilibrium concentration of that volume element a time t_r previously. At that time the volume element was a distance vt_r upstream. However, as the volume element moved downstream from this point a distance vt_r, the overall concentration, which determines the equilibrium, moved the above quoted distance of Rvt_r. The distance between these displacements, $vt_r\,(1 - R)$, becomes, in this formulation, the distance from the midpoint to the end of the cell.

$$\frac{(\text{HETP})}{2} = vt_r\,(1 - R) \tag{26}$$

In this case, then

$$(\text{HETP}) = 2vt_r\,(1 - R), \qquad N = t_1/2t_r\,(1 - R) \tag{27}$$

and for the A $\rightleftarrows$ B model, with $t_r = 1/(k_1 + k_2)$ and $R = k_2/(k_1 + k_2)$, we have

$$(\text{HETP}) = 2k_1v/(k_1 + k_2)^2, \qquad N = (k_1 + k_2)^2\,t_1/2k_1 \tag{28}$$

The latter expression results when the half-width for the continuous-flow model is compared to that from stochastic theory. We have used an expression derived by SAID[16] for this comparison.

DISCUSSION

The foregoing treatment relates both an effective diffusion coefficient and the number of theoretical plates to the kinetics of sorption and desorption. For practical purpose, it is necessary that these parameters be related to experimental procedure. This matter has been extensively discussed in the case of the number of theoretical plates[17].

It is common practice to inject a sample into a chromatogram with the least possible spread. In the limit we may consider all molecules started simultaneously in the column. When, in addition, near-equilibrium conditions prevail, the concentration profile becomes Gaussian, both when the component is spread as a band on the column, and in the column effluent. A measure of the diffusion effect is the standard deviation of this concentration profile. In general a procedure will become more valuable as the ratio of the standard deviation to the displacement from the origin (referred to the center of the band) becomes smaller[8]. With a component still on the column, the standard deviation is $\sigma = \sqrt{2Dt}$ (for any diffusion process), and the distance of the band from the origin is $\bar{z} = \bar{u}t = Rvt$. Substituting in the appropriate values from the kinetic analog, (2), we find that the dimensionless ratio, $\lambda = \sigma/\bar{z}$, is

$$\lambda = \left(\frac{2k_1}{k_2\,(k_1 + k_2)\,t}\right)^{1/2} \tag{29}$$

In the case of elution, the standard deviation is usually measured in terms of time. The displacement from the origin, the elution time, t, is of the same dimensionality. For the kinetic analog, (2), the former is[5]

$$\sigma = (2k_1Rt)^{1/2}/k_2 = [2k_1t/k_2(k_1 + k_2)]^{1/2} \qquad (30)$$

and the ratio, $\lambda = \sigma/t$, is identical to (29).

The value of λ can be easily obtained for the theoretical-plate model. Substituting $t_1 = Rt$ into (28) and comparing with (29), we find, $\lambda = 1/\sqrt{N}$.

With the foregoing results, and the experimental values for both λ and R, it is easy to determine the kinetic rates of transition, k_1 and k_2. It is then possible to study the dependence of the transition rates upon temperature, solvent and sorbent properties, etc. Such a study shows promise of additional control over the movement of chromatographic zones.

SUMMARY

It has been the object of this presentation to show connecting links between the various theories of chromatography. The stochastic theory, the material-conservation approach, and the theoretical-plate model are treated individually and in relation to one another. The last two are treated as problems in non-equilibrium kinetics, exemplifying the concept that it is the lack of equilibrium between the mobile and stationary phases that causes the smearing of individual solute bands. The source of the non-equilibrium, as well as the smearing effect due to non-equilibrium, are discussed both qualitatively and quantitatively. The quantitative treatment of these cases depends on the use of the kinetic relaxation-time model, originally devised for the study of non-equilibrium kinetics in the flame front.

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A GENERAL METHOD FOR DETECTION AND RECORDING OF COMPONENT BANDS IN CHROMATOGRAPHY WITH LIQUID ELUENTS

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INTRODUCTION

A promising general method has been developed for the detection and continuous recording of component bands in chromatographic separations employing liquid eluents (paper, string or packed column chromatography). Since the method is based primarily upon colligative, rather than structural, properties, its applicability is not limited to specific classes of components or eluents.

PRINCIPLES

The method utilizes measurements of the temperature differential which is established between a cellulose wick saturated with the pure eluting solvent and a similar wick which is continuously bathed with the liquid effluent from the chromatographic column; both wicks are enclosed in a thermostatted chamber saturated with solvent vapor. The use of thermistors as the temperature-sensing elements permits use of a conventional Wheatstone bridge circuit, which can be balanced when both wicks are bathed with pure solvent. Since a small electrical current passes continuously through the thermistors in the bridge circuit, the temperatures sensed by the thermistors depend primarily upon competition between the following processes of heat input and heat removal.

1. Heat input:
   a. Electrical heating of the thermistor.
   b. Heat evolved by condensation of solvent on the wick.
2. Heat removal:
   a. Thermal conduction of heat to the liquid flowing through the wick.
   b. Heat absorbed in vaporization of the solvent (and, possibly, volatile solutes) from the wick.

The electrical heating and liquid flow are held essentially constant. When the column effluent liquid contains solute in a component band, the thermal conductivity of the eluting solvent is not significantly altered by the presence of solute. The vapor pressure of solvent, however, is lowered by the presence of solute, causing the ratio of the rate of vaporization to the rate of condensation to become lower on the sample wick than on the reference wick; the sample thermistor thus seeks a new thermal balance point corresponding to a higher temperature. When the bridge unbalance is fed directly across the input terminals of a recording potentiometer, a peak is recorded for each component band eluted from the column. (If the component is more volatile than the solvent, the new balance point for the sample thermistor is at a lower temperature, rather than a higher temperature, through evaporative cooling.)

EXPERIMENTAL

The experimental arrangement of the detector is shown schematically in Fig. 1. The detectors, T_r and T_s, are glass-enclosed, 2000 Ω, bead-type thermistors (randomly selected, not matched) with a response of about 5% per degree Centigrade. The bridge circuit includes a 4000 Ω fixed resistor, R, in one arm and a decade resistance box in the other. The decade box possesses dials for thousands, hundreds, tens, and units (Ω). The bridge is operated at a 3 V potential furnished by dry cells. When using a 1-mV full-scale recording potentiometer, it is possible to obtain a deflection of 12% of full scale for a 1 Ω change in the setting of the decade resistance box; this deflection corresponds to a temperature differential of about 0.01° between the two thermistors as compared to the balance condition. Thus changes of the order of 0.001° can be clearly seen, and even a differential of only a few ten-thousandths of a degree can be detected when the background signal is sufficiently stable.

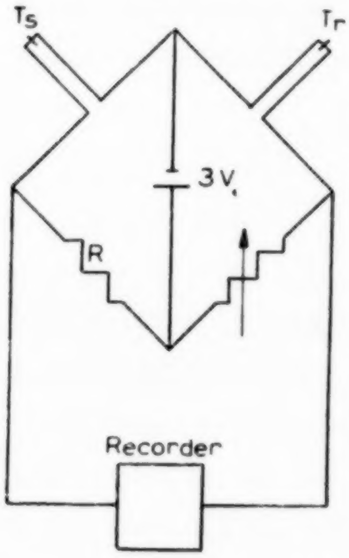

Fig. 1. Experimental arrangement of detector (schematic).

Greater sensitivity could be obtained by use of a higher bridge voltage; a factor of three could be gained in this manner by operating at 9 V, but still higher voltages would shorten the detector life. The limiting factor at present seems to be thermostatting, rather than detector sensitivity, although use of carefully matched thermistors would decrease the background signal due to bath temperature fluctuation. In the preliminary studies carried out to date, no attempt has yet been made to achieve the ultimate possible sensitivity.

The sample and reference chromatographic columns are water jacketed with force flow of jacket water from the constant temperature bath (the jacketing is essential only on the lower portion of the column, so that longer columns could be used). The columns are mounted directly above the detector, which is contained in a large glass test tube immersed in the thermostatting bath. The effluent from the two columns flow into two separate small-diameter siphon tubes, which carry the liquid out of the thermostat into collecting vessels. Solvent in the sample column is forced in from a reservoir which maintains a constant head of gas pressure above the liquid. Sample is injected into the top of the column by means of a long hypodermic needle through a three-way stopcock which connects the solvent reservoir to the column and the outside. Many modifications of the columns, solvent feed, and sample injection are possible; the method which was used in this study has been indicated only to show the type of framework in which the detector has been utilized.

A minor modification of the experimental arrangement makes possible the recording of the derivative curves for the component peaks rather than the direct recording of the peaks themselves; this is achieved by placing both thermistors on the wick from the sample column, separated by some selected distance. When a non-volatile component first reaches the upper thermistor a deflection is obtained corresponding to its temperature rise; as the component band reaches the lower thermistor, the differential temperature recorded is a measure of the composition difference between the locations corresponding to the two thermistors. If the distance between thermistors is small relative to the width of the component band, the recorded differential temperature is nearly an instantaneous derivative of the band shape, showing a deflection in one direction as the beginning of the band reaches the upper thermistor, zero deflection as the peak passes a point halfway between the thermistors, and a deflection in the other direction as the tail of the band passes the lower thermistor. The potential advantage in the derivative method lies in its greater sensitivity in detecting poorly resolved component peaks; this sensitivity arises from the more detailed structure of a derivative curve as compared with the direct curve.

RESULTS

The response of the detector has been investigated for a variety of solutes in three solvents: water, butanol saturated with water, and 95% ethanol. The detector has been found to respond to each solute tested. For solutes of higher volatility than the solvent, or capable of forming with the solvent, azeotropes more volatile than the solvent, the response indicates a lowering of temperature of the sample thermistor relative to the reference thermistor. For solutes of lower volatility than the solvent,

TABLE I

RESPONSE OF DETECTOR TO VARIOUS SOLUTES

	Boiling point, °C	*Direction of deflection with water as solvent*	*Remarks*
Glycerol	290	+	Also + with butanol–water and 95% ethanol as solvents
Ethylene glycol	197.4	+	
Butanol-1	117	—	Forms azeotrope with water, b.p. 92.7°
Propanol-2	82.5	—	
Ethanol	78.4	—	Also — with butanol–water as solvent
Chloroform	61.2	—	
Acetone	56.5	—	
Benzene	80.1	—	
Acetic acid	118.1	+	
Sugar	non-volatile	+	
Food colors	non-volatile	+	
Ink	non-volatile	+	
Salts (NaCl, NaBr, NaI, KCl, KBr, LiCl, $CuCl_2$)	non-volatile	+	

including non-volatile solutes, the response indicates an increase of temperature of the sample thermistor relative to the reference thermistor. Some of the results obtained are tabulated in Table I, in which a temperature increase of the sample thermistor is indicated by "+" and a temperature decrease by "—".

Examples of recorded chromatograms obtained with detector are shown in Figs. 2 and 3.

The actual magnitude of the response has been found to depend upon several factors, making comparisons of the response for different solutes relatively qualitative at the present time. The maximum response which has been observed thus far has been for a system employing 95% ethanol as eluent and with the detectors mounted on cotton string wicks. With this system, a sample of 2 μl of ethylene glycol gave a peak height corresponding to 50 Ω, corresponding to a temperature differential of about 0.5°. Smaller responses result from the use of less volatile solvents and larger wicks.

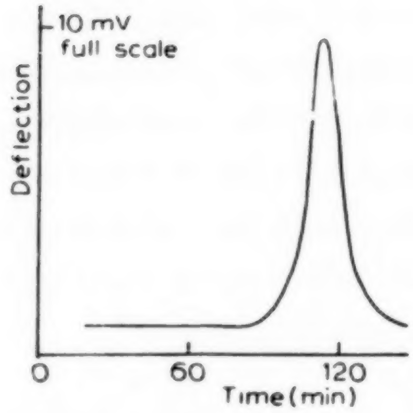

Fig. 2. 0.1 ml of 10% ethylene glycol.

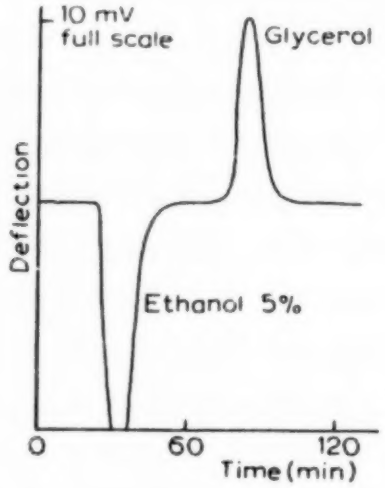

Fig. 3. 0.2 ml sample showing opposite deflection of volatile components.

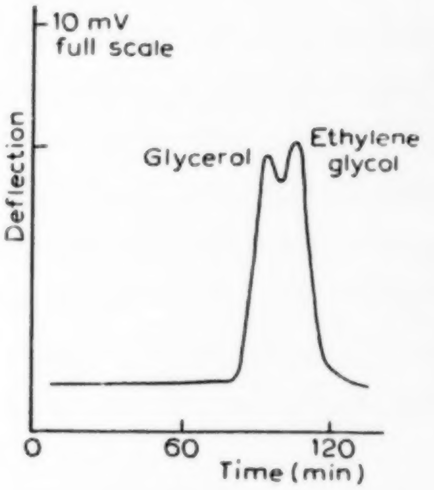

Fig. 4. 0.2 ml sample showing some resolution.

The factors which have been found to be important in determining the response are:

(1) the size of the sample,

(2) the rate of flow through the column,

(3) the size of the detector wick,

(4) the nature of the solvent, and (in the case of volatile solutes) the nature of the solute.

Each of these factors will now be considered briefly.

1. *Sample size*

The response of the detector has been found to vary directly with sample size for pure liquid samples and with sample concentration for samples introduced as solutions in the eluting solvent, with all other conditions held constant. A study of equally sized samples of a series of concentrations of aqueous solutions of sodium chloride demonstrated that both peak heights and peak areas varied directly with concentration.

2. *Flow rate*

The rate of flow of solvent through the detector wick is, unfortunately, a variable to which the quantitative response of the detector is extremely sensitive. Since the concentration of solute bands tends to be decreased at higher flow rates, and the solute spends less time in contact with the thermistor, peak heights and peak areas are both decreased by increasing flow rate. For comparison of results of different runs, close control of this variable is essential; better comparisons are obtained for different components in a single run.

3. *Size of wick*

The effect of this variable is closely dependent upon the flow rate through the column. The wick should be large enough that it does not "flood" in handling the flow rate used; drops of free liquid flowing over the detector do not permit sufficient equilibration of the liquid and vapor phases at the thermistor. On the other hand, if the wick is too large dilution of the sample bands may occur, decreasing the detector response. Both cotton string and cotton plug wicks have been used in this work.

4. *Nature of the solute and solvent*

While extensive quantitative data have not yet been obtained, it appears that the response to non-volatile solutes is essentially a colligative property, independent of the nature of the solute. This behavior is to be anticipated on the basis of the postulated mechanism for the detector response. The cooling of the sample thermistor observed for volatile solutes of higher volatility than (or forming more volatile azeotropes with) the solvent is in agreement with an explanation based upon relative vapor pressure lowering as the colligative property, modified by kinetic effects due to the non-equilibrium nature of the system.

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SUMMARY

A method for the detection and recording of component bands has been described. It was found to be sufficiently general to insure its applicability to a wide variety of solvent–solute systems, and since it senses the column effluent it is independent of the type of chromatographic column used. It appears to be readily adaptable to the quantitative estimation of non-volatile solute bands for which it is based on essentially a colligative property, and it appears applicable, with specific calibration, to volatile solute bands as well.

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A SEMI-AUTOMATIC MULTI-PIPETTING DEVICE FOR PAPER CHROMATOGRAPHY

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INTRODUCTION

The smaller the area of the spot of solution applied to the paper in partition chromatographic technique, the more sharply defined are the separated spots after developing the chromatogram (CRAMER[1]); except in special circumstances not more than 10 μl should be applied at a time and usually it is recommended that 2–5 μl be used (CRAMER[1]; BLOCK, LESTRANGE AND ZWEIG[2]). For this purpose various glass pipettes made to deliver volumes of the above order have been described (see, for example, ISHERWOOD AND HANES[3]; BRIMLEY AND BARRETT[4]).

Where the concentration of materials to be detected or estimated is low it may be necessary to apply the equivalent of 50–200 μl of solution to the chromatogram in order to detect the separated materials. Two approaches are then available: (a) to concentrate the original solution before application to the paper; (b) to apply the total volume of original solution necessary, 2–5 μl at a time, drying each spot completely before applying the next one (CRAMER[1]; BLOCK, LESTRANGE AND ZWEIG[2]).

Proceedure (a) may be technically difficult if for example it is necessary to concentrate an already small sample to say 100 μl to achieve the necessary concentration; it may also be time-consuming and inconvenient if there are a number of samples to be handled.

Procedure (b) is technically more elegant but extremely tedious with the usual simple pipetting apparatus and where more than one solution has to be analysed the time taken in spotting can become prohibitive. LEDERER AND LEDERER[5] refer to several methods in the literature designed to apply large volumes or large masses of material to chromatograms as spots or streaks. Of the methods quoted, only those of URBACH[6], of YANOFSKY, WASSERMAN AND BONNER[7] and of GLAZKO *et al.*[8] appear to be capable of quantitative transfer of solution to the paper, and none of the apparatus quoted appears to be as versatile as the present device although in its current form it cannot apply solution as a streak to the paper; a modification to perform this task is being developed.

The apparatus described here (Fig. 1) eliminates the tedious features of the repeated spotting technique and is so designed that any given volume of the same or of different solutions can be applied to each of as many as twelve separate starting points on the chromatogram(s) in the time taken to apply and dry the same volume on one starting point using the usual techniques.

CONSTRUCTION

The apparatus (Fig. 1) consists of two main parts:

A. The paper holder

This is made from a single sheet of aluminium cut and bent to the shape and dimensions shown in Fig. 2b and rendered rigid by screwing to each side a piece of wood cut to the profile of the paper holder (Fig. 3). The depth of the holder suits laboratory benches and the quarter-circle upward bend at the back keeps long papers clear of reagent galleries etc.; the inclined surface at the front of the holder raises the chromatography paper so as to permit contact between the paper and the delivery tips of the pipettes (Figs. 1 and 3).

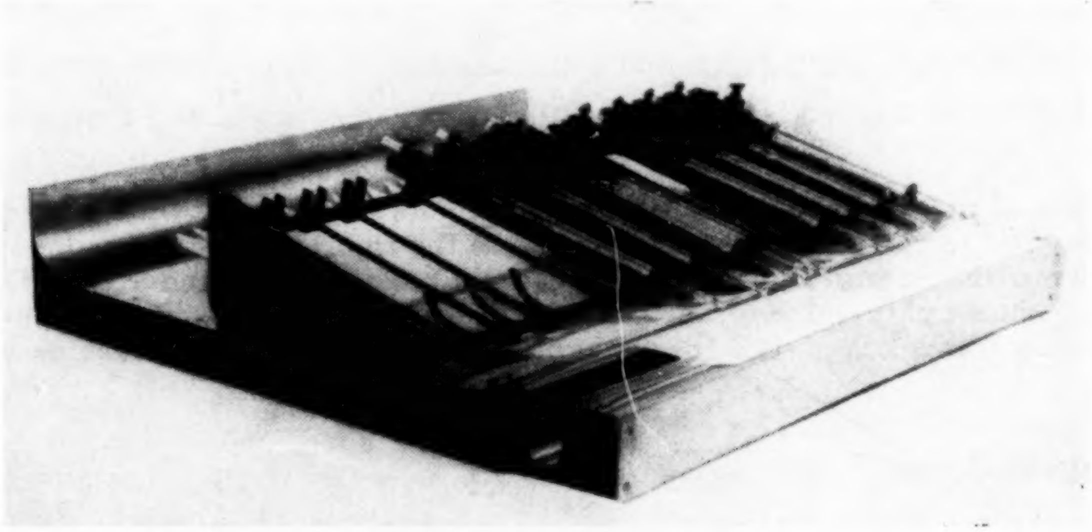

Fig. 1. The complete multi-pipetting device in use.

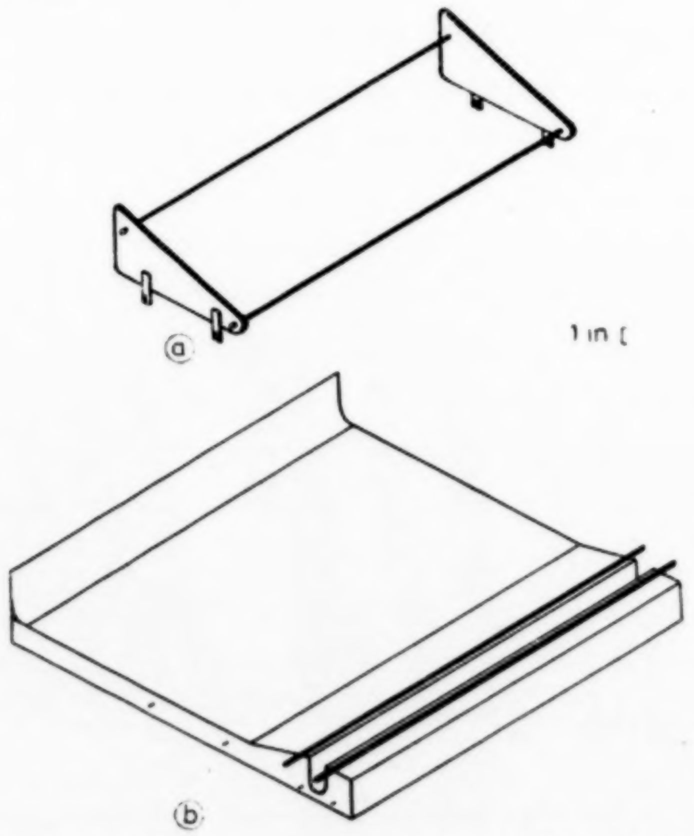

Fig. 2. (a) The carrier frame above (b) the paper holder onto which it is located when in use; the glass rods for clamping the paper are shown on either side of the channel down which air is blown to dry the solutions applied to the paper. (1 in. = 2.54 cm).

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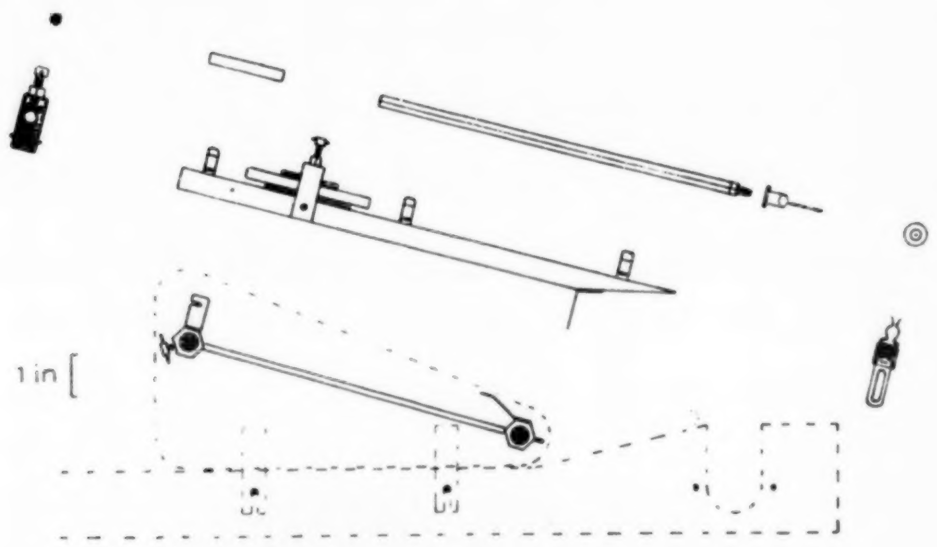

Fig. 3. Side view of the pipetting device ready for assembly: reading downwards—a pipette (with its glass sealing rod to the left and syringe needle to the right); a pipette-carrier with rubber air reservoir between the compression plates; and a slide. To the left and right of the respective components are shown part end views of the pipette and of the pipette-carrier. The triangular side plate of the carrier frame and part of the paper holder are shown as dotted lines. (1 in. = 2.54 cm.)

B. The pipetting device

This locates onto the paper holder in the manner shown in Figs. 2 and 3; it is shown removed from the paper holder in Fig. 4. The components of the pipetting device are as follows:

(i) *The carrier frame* (Fig. 2a) which consists of two triangular side plates held rigidly apart by two stainless steel rods which also serve as guide rods for the slides described below.

(ii) *A series of slides* (Fig. 3), each made from a pair of hexagonal-section brass bushes soldered onto the opposite ends of a brass connecting rod; the bushes are a sliding fit on the steel guide rods of the carrier frame to which an appropriate number of the slides are permanently fitted (Fig. 4). Figs. 5a and 5b show the detailed construction and ancillary fittings of the rear and front bushes respectively of one slide.

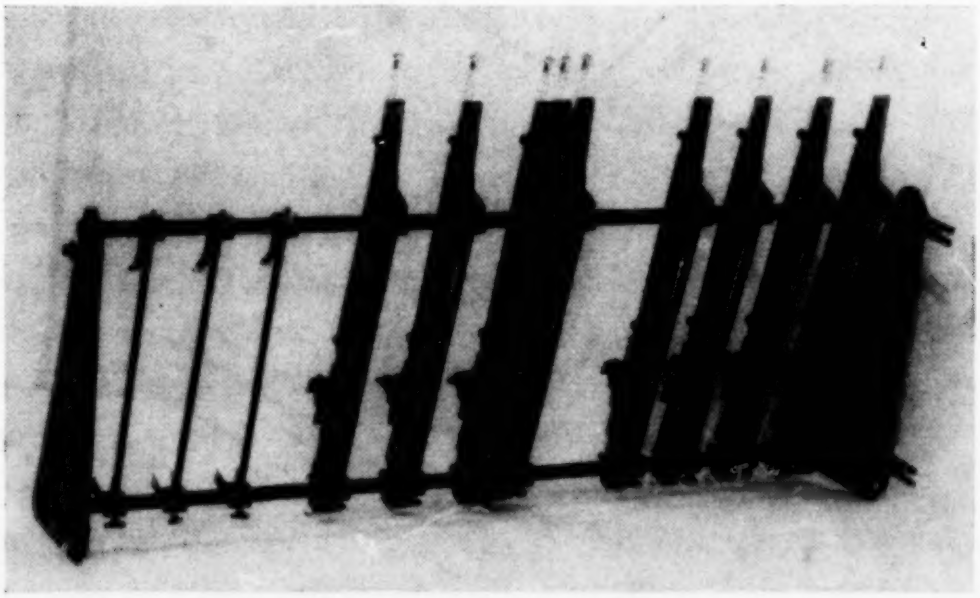

Fig. 4. A view from below of the pipetting device illustrating the variation in lateral separation of the pipettes which is possible on the carrier frame. Of the twelve slides on the carrier frame nine are fitted with pipette-carriers and pipettes.

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(iii) *A series of pipette-carriers*, one for each slide, are made from lengths of square-section hardwood (Fig. 3). Near the back of each carrier, a brass pin is forced through a hole drilled horizontally through the wood; the pin projects on both sides of the pipette-carrier and is a neat fit in the slot of the rectangular channel fitted to the rear bush of a slide (Figs. 3 and 5a). Near the front of each pipette-carrier is fixed a slotted brass strip in such a position that with the carrier riding in the channel on the rear bush of a slide, this slotted strip has to be sprung slightly to pass over the pin screwed into the front bush of the slide (Figs. 3 and 5b); this pin in the front bush then engages in the slotted strip which springs back against the front bush. Lateral or longitudinal movement of the pipette-carrier is now impossible but it can be depressed with a radial motion against the clock spring attached to the top of the front bush of the slide. Three spring clips are fixed to the upper surface of each pipette-carrier (Fig. 3). The compression device for operating the air reservoir of a pipette is illustrated *in situ* in Fig. 3; the details of its construction and the method of fixing it permanently to the pipette-carrier are shown in Fig. 5c.

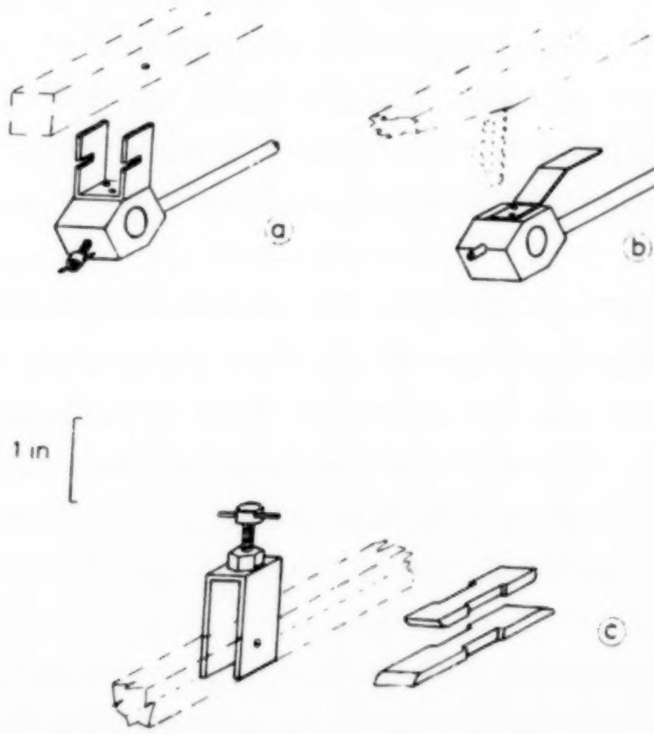

Fig. 5. (a) Detail of rear bush of a slide showing the slotted channel (into which fits the pipette-carrier) and the locking-screw for fixing the slide in position on the carrier frame (see Figs. 2 and 3). (b) Detail of front bush of a slide showing the spring (on which the front end of the pipette-carrier rests) and the pin which engages with the slotted strip projecting below the front of the pipette-carrier (see Fig. 3). (c) Detail of the inverted brass channel and the compression plates which operate the pipette (see Fig. 3). (1 in. = 2.54 cm.)

(iv) *A series of pipettes:* for qualitative work these are made from lengths of approximately 1 mm bore capillary tubing to one end of which is sealed a standard glass male joint such as that found on hypodermic syringes (the joints are obtainable from hospital supplies stores); a No. 20 "Record" stainless-steel hypodermic needle fitted to the male joint provides a robust and removable delivery tip through which the pipette delivers 5 μl accurately. The pipettes are each completed by an air reservoir of seamless rubber tubing sealed with a glass rod (Fig. 3). The pipettes are calibrated roughly by any convenient method. For quantitative work the capillary tubing is

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replaced by commercial pipettes graduated in 0.005 ml divisions from 0 ml on the stem to 0.1 ml at the shoulder; the delivery tip is cut off well away from the 0.1 mark and a standard male joint sealed on in its place.

OPERATION OF THE APPARATUS

A sheet of chromatography paper is sandwiched between two sheets of grease-proof paper, leaving a 10–15 cm strip projecting from the sandwich; across this strip are marked the starting line and starting points for the application of the solutions to be analysed. The sandwich of papers is laid flat on the paper holder with the starting line over the U-channel and the grease-proof papers as close as possible to the back edge of the U-channel; the papers are clamped by glass rods (Figs. 1, 2b and 3) and rubber bands stretching from the rods to small hooks in the wooden sides of the paper holder.

The carrier frame (Fig. 2a), complete with the appropriate number of slides (Fig. 3), is placed on the paper holder (Fig. 2b); the lower part of Fig. 3 shows the arrangement at this stage.

If rapid drying of spots of solution on the chromatography paper is required, a domestic hair-dryer is placed at one end of the U-channel in the paper holder (Figs. 2 and 3) so that hot or cold air can be blown down the channel.

A pipette, complete with air reservoir and sealing rod, is clipped into each pipette-carrier by means of the spring clips on the carrier (upper part of Fig. 3); the male joint of each pipette is lightly greased and a hypodermic needle (oblique surface downwards) is fitted to each pipette.

A pipette, on its carrier, is held vertically and the needle inserted below the surface of the solution to be analysed; air is ejected from the pipette by screwing down the thumb-screw (Figs. 3 and 5c); solution is drawn up above the zero mark by unscrewing the thumb-screw. The pipette tip is removed from the solution and liquid slowly ejected to the zero mark.

The pipette-carrier, complete with the filled pipette, is now fitted into one of the slides on the carrier frame in the manner described under CONSTRUCTION, section (iii). The slide, now complete with its pipette-carrier and pipette, is moved across the carrier frame until the pipette tip corresponds with the appropriate starting point on the chromatography paper (Figs. 1 and 4); the slide is locked in this position by means of the locking-screw in its rear bush (Figs. 3 and 5a). The delivery tip of the pipette should now be about 10 mm above the chromatography paper.

The filling and mounting of the remaining pipettes is completed serially, when the apparatus will appear as in Fig. 1.

The first pipette of the series mounted on the pipetting device (Fig. 1) is now depressed against the clock spring on its slide (Fig. 3) so that the delivery tip makes contact with the starting point on the chromatography paper; 2–5 μl of solution is squeezed out by turning the thumb-screw. When the appropriate volume has been delivered the pipette is released and springs away from the paper, thus avoiding capillary extraction of more than the required volume of fluid from the pipette. The operation is repeated with each pipette in turn. Using twelve pipettes, the solution applied by the first is usually dry by the time the twelfth pipette is operated; the first pipette can therefore be operated again immediately after the twelfth. The operations are repeated serially until the required total volume of solution has been applied to each starting point on the chromatogram. It is usually found that capillary action by the paper will extract a total of approximately 20 µl solution from a pipette once the flow has been started by turning the thumb-screw slightly.

FEATURES OF THE ASSEMBLED APPARATUS (Fig. 1)

Each pipette is fitted to an independent pipette-carrier (Fig. 3) from which it is quickly detached and dismantled for cleaning without disturbing the rest of the apparatus; each pipette-carrier is attached to one of a series of independent slides (Fig. 3) which can be moved laterally across a carrier frame (Figs. 2a and 4) to correspond with any desired point of application on the chromatography paper (Fig. 1). A maximum of 24 pipettes can be accommodated at 2 cm centres across a 56 cm paper, allowing a 5 cm margin at each side; a more useful arrangement is a series of 10 pipettes which can then be set so that the distance between pairs of pipette tips is as little as 2 cm or as great as 5 cm, assuming equidistant starting points and 5 cm margins at either side of the paper. Any pipette-carrier, complete with its pipette (Figs. 3 and 4) can be removed individually from its slide on the assembled apparatus (Fig. 1) at any time to refill the pipette without disturbing the rest of the apparatus. Quantitative transfer of any volume of solution to the paper is achieved. Provision is made for hot or cold air drying of the solution applied and once a paper is "spotted" it can be removed and replaced by another paper (without disturbing the pipettes), by releasing the clamping rods (Figs. 1, 2b and 3) and sliding the sandwich of papers out. Any length of paper can be used and any width up to 60 cm. The apparatus is equally useful for applying solutions to each of several paper strips for one-dimensional chromatography, or for applying solution to a single starting point on each of four 28 cm × 23 cm papers for two-dimensional chromatography; in the latter instance the papers are placed diagonally on the paper holder so that the starting point near the corner of each sheet does not overlap an adjacent sheet.

It is obvious that at least one of three minimum conditions must apply before the apparatus becomes useful:

(i) More than about 10 applications of 2–5 µl of solution are required to place sufficient material on a single starting point.

(ii) A number of different solutions or of replicates of the same solution require to be spotted, each solution requiring more than 4 or 5 applications of 2–5 µl.

(iii) It is desired to replicate the application of relatively small volumes of several solutions on several sheets of paper.

When one or more of these conditions apply, the apparatus should be equally useful in research or routine analytical use, since it takes only about 20 minutes to set up the apparatus and fill 12 pipettes before applying the solutions to the chromatography paper; thereafter operations are extremely rapid. In the author's laboratory the apparatus has been successfully used for the application of 80–100 μl samples of solutions requiring to be analysed for various carbohydrates (CHRISTIE AND PORTEOUS[9]) by the techniques of BEALING AND BACON[10].

ACKNOWLEDGEMENTS

The apparatus described here was constructed in the departmental workshop by Mr. A. G. SILVER whose work the author is glad to acknowledge.

I wish also to thank JAMES JAMIESON for the photographs reproduced as Figs. 1 and 4.

SUMMARY

A semi-automatic multi-pipetting device is described which has proved useful in paper chromatography, particularly where it has been necessary to apply large volumes of a solution or a large number of different solutions to one or more chromatographic papers for one- or two-dimensional development. Any length of paper and any width up to 60 cm can be accommodated; the distance between starting points can be varied at will within wide limits; pipettes can be removed individually at any time for cleaning or refilling and the papers can be changed without disturbing the rest of the apparatus; provision is made for hot or cold air drying of the solutions applied to the paper.

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GAS CHROMATOGRAPHY OF POLAR COMPOUNDS USING A NON-POLAR LIQUID PHASE

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INTRODUCTION

The examination of strongly polar solutes by gas–liquid chromatography (G.L.C.), using non-polar liquid phases, invariably results in poor separations, due to the adsorption of the polar compounds on the surface of the support. This adsorption effect manifests itself by the production of asymmetric peaks with sharp "fronts" and very diffuse "tails". Both Celite and ground insulating-brick, the supports commonly used in G.L.C., exhibit this effect, although the adsorption that occurs with Celite can be reduced slightly by treatment with acid and alkali.

This adsorption effect has been overcome by Sörensen and Söltoft[1] and by Kwantes and Rijnders[2], by using metal helices as the support. Symmetrical peaks were obtained but the column resolution was poor due to the open nature of the support allowing considerable molecular diffusion[3] to take place in the column. The work of these authors indicated that a support with a metallic surface was preferable for use in G.L.C. and this paper describes a method for coating brick dust with both gold and silver deposits. Preliminary work with filings from a porous phosphor-bronze bearing as a support certainly produced symmetrical peaks with little or no tailing but the metal was insufficiently porous to hold a reasonable quantity of liquid phase and this support was discarded.

EXPERIMENTAL

Johns-Manville C.22 insulating-brick, ground to a particle size of 120–160 B.S. mesh, was used as the support. It was degreased with chloroform prior to the deposition of the metal. Silvering of the prepared brick dust was then carried out using the "Rochelle Salt" process[4]. Ten grams of the brick dust were treated with 100 ml of the silvering solution and degassed under reduced pressure. This ensured that the solution penetrated into the pores of the brick. One hundred ml of the reducing solution was then added, and the solution was stirred for approximately 10 minutes. Care was taken to avoid vigorous stirring, in order to eliminate possible reduction of the particle size of the support. The surplus liquid was decanted from the support and a further 100 ml of silvering solution added. This process was repeated for two

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separate 10 g portions of brick dust until they had been coated with 4 g and 10 g of silver respectively.

The gold plating of the degassed brick dust was carried out using a solution of brown gold chloride in absolute alcohol. Sufficient solution, containing 1 g of gold chloride, was added to the brick dust to form a slurry and the mixture degassed. The alcohol was then removed in a gentle stream of warm air and the brick dust heated to 250° in a slow stream of hydrogen. The hydrogen was passed through the brick dust until no acid reaction was obtained from the exit gas. This procedure was repeated ten times, giving successive deposits of gold on the support, until a total of 6.5 g of gold had been deposited. Examination of the coated brick dusts under the microscope showed a uniform covering of silver, but only a very patchy coating of gold.

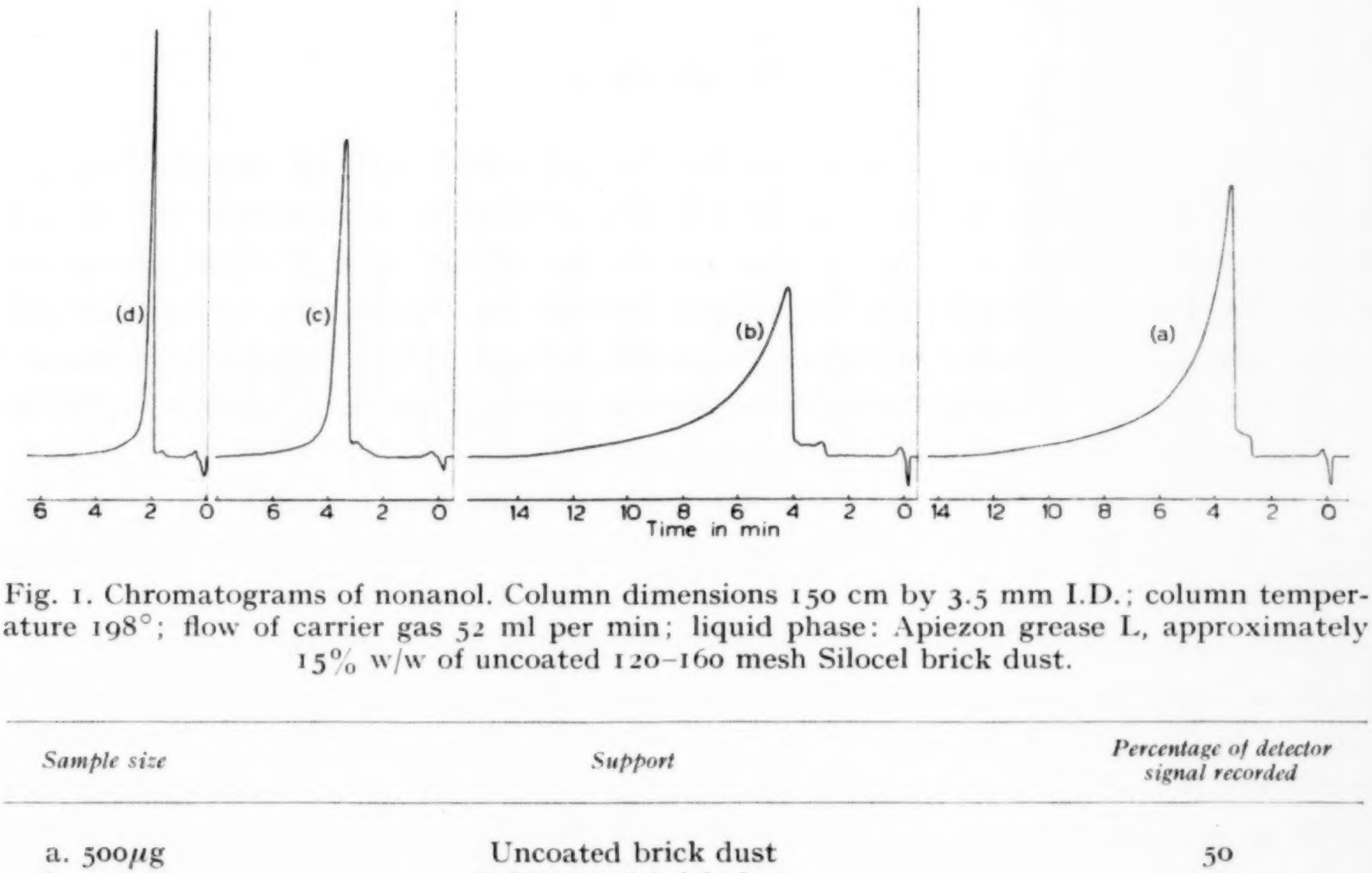

Fig. 1. Chromatograms of nonanol. Column dimensions 150 cm by 3.5 mm I.D.; column temperature 198°; flow of carrier gas 52 ml per min; liquid phase: Apiezon grease L, approximately 15% w/w of uncoated 120–160 mesh Silocel brick dust.

Sample size	*Support*	*Percentage of detector signal recorded*
a. 500 μg	Uncoated brick dust	50
b. 500 μg	Gold coated brick dust	50
c. 300 μg	Silver coated brick dust 3.9:10 silver to brick	20
d. 300 μg	Silver coated brick dust 9.5:10 silver to brick	20

After degassing the coated support, the liquid phase (Apiezon L grease) was placed on it in the usual manner. The ratio of liquid phase to support, based on the weight of the original uncoated support, was 15% w/w. To obtain comparative results a sample of uncoated support was also prepared. The supports carrying the liquid phase were packed in a 150 cm column 3.5 mm I.D. and run at 198° in an apparatus utilizing the flame detector. A number of polar substances were examined and two sets of chromatograms obtained are shown in Figs. 1 and 2.

RESULTS AND DISCUSSION

The chromatograms shown in Fig. 1 for the gold-coated support show little improvement on the uncoated brick dust, whereas the silver coating almost eliminates the asymmetry of the nonanol peaks. The inability of the gold deposit to produce symmetrical peaks was thought to be due to its irregular deposition. The improved separations obtained by reduction of the asymmetry caused by adsorption is shown in Fig. 2. A 500 μg sample of impure methylcyclohexanol was injected to produce the first two chromatograms (a) and (b) and it will be seen that some impurities were present adjacent to the major peak. A 300 μg charge was sufficient to produce the chromatogram (c) and the reduction in asymmetry resulted in two of the three apparent impurities being partially separated from the main peak. In the chromatogram (d) further decrease in adsorption resulting from increased silver coating has resulted in even better separation between the second and third peaks. The spread of the original peak was in no way due to overloading of the column and the reduction in charge was necessary because the peaks were narrower and, therefore, higher in form. There are, however, some disadvantages in using metallic silver as a coating for the support. Sulphur compounds tend to react with the silver and difficulty has

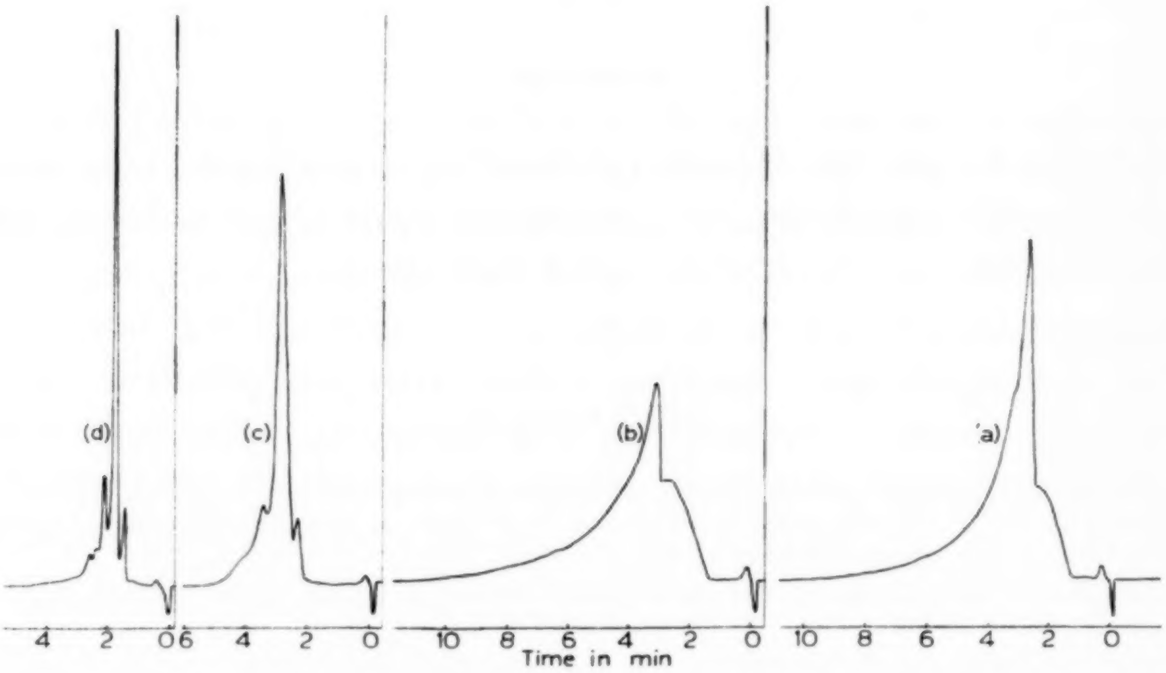

Fig. 2. Chromatograms of methylcyclohexanol. Column dimensions 150 cm by 3.5 mm I.D.; column temperature 198°; flow of carrier gas 52 ml per min; liquid phase: Apiezon grease L, approximately 15% w/w of uncoated 120–160 mesh Silocel brick dust.

Sample size	*Support*	*Percentage of detector signal recorded*
a. 500μg	Uncoated brick dust	50
b. 500μg	Gold coated brick dust	50
c. 300μg	Silver coated brick dust 3.9:10 silver to brick	20
d. 420μg	Silver coated brick dust 9.5:10 silver to brick	20

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been experienced when chromatographing amines such as diethylamine and diethylaminoethanol. Further improvements in the coating of the support to eliminate these effects might be obtained by coating gold on top of silver or possibly by using nickel coatings from the decomposition of nickel carbonyl[5].

ACKNOWLEDGEMENTS

The authors wish to thank Mr. G. S. F. HAZELDEAN for his help with the experimental work and the directors of Benzole Producers Limited for permission to publish these results.

SUMMARY

A reduction in the asymmetry of peaks produced by strongly polar compounds during gas chromatographic examination on a non-polar liquid phase has been achieved by using Johns-Manville C.22 brick dust coated with silver.

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THE EFFECT OF PRESATURATION OF THE PAPER ON THE SEQUENCE OF Bi–Cd IN PAPER CHROMATOGRAPHY WITH BUTANOL–*N* HCl

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In carrying out preliminary work with a Craig-type counter-current apparatus we thought it of interest to separate a mixture of Cu–Cd–Bi with butanol–*N* HCl and observe the sequence under true equilibrium solvent extraction conditions, since different sequences have been reported[1,2].

It was found that the sequence obtained in a counter-current apparatus was identical with that obtained with ascending paper chromatography, as shown in Table I. Here "R_F" values are obtained by analysing each tube by paper chromatography and expressing the movement of the centre of a band with relation to the movement of the pure solvent as R_F value.

TABLE I

	20 tubes	*40 tubes*	*60 tubes*
R_FCd	0.27	0.25	0.23
R_FBi	0.38	0.35	0.33

These findings incited us to investigate the variables that change the sequence of Bi–Cd under certain conditions in paper chromatography. The question was considered of some importance because Lederer[1] used ascending development in large jars, whereas Burstall *et al.*[2] employed descending development in a narrow cylinder holding a small solvent compartment only. The possible influences which were considered were thus descending or ascending development and efficient saturation of the atmosphere.

We first investigated the last-mentioned effect, having had some experience with the separation of curare alkaloids, where presaturation of the paper is essential to prevent comets. We thus spotted mixtures on sheets of Whatman No. 1 paper and allowed them to stand for different lengths of time to reach equilibrium with the atmosphere of the solvent jar, before starting actual development.

A remarkable effect on the R_F values and the sequence of Bi–Cd in butanol–*N* HCl was noted. Without saturation the sequence according to Burstall *et al.*[2], *i.e.* Cd preceding Bi, was found. As soon as the paper was equilibrated for more than 24 hours this was reversed, as shown in Table II.

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TABLE II

Saturation (days)	*Distance travelled by the solvent (cm)*	*Distance travelled by the wet front (cm)*	R_F *Cd*	R_F *Bi*	R_F *wet front*
0	16	9.5	0.52	0.46	0.59
0	17.8	11	0.54	0.50	0.63
1	19.5	10.5	0.38	0.45	0.50
1	18.7	9.7	0.37	0.45	0.52
2	18.4	9.3	0.37	0.46	0.50
2	17.4	9.5	0.38	0.49	0.545
3	16.6	8.8	0.38	0.47	0.53
3	18.8	9.2	0.34	0.44	0.49
7	18	9.6	0.39	0.47	0.53

We then developed chromatograms saturated for 24 hours both by the ascending and descending method in the same container and found no notable differences in the R_F values and sequences (Table III).

We therefore suggest that the discrepancy of results is due to the fact that the work of LEDERER[1] was carried out in the rather warm climate of Sydney, Australia, which sufficiently increased the equilibration rate to make presaturation unnecessary.

TABLE III

	Ascending technique	*Descending technique*
R_FBi	0.46	0.44
R_FCd	0.38	0.37

We would further like to point out that equilibration does not improve the separation of Cd–Bi. However, this factor becomes important when exact R_F values are required as in some physico-chemical calculations.

The data obtained by our counter-current extraction experiments lend themselves to the calculation of the ratio of the two phases on the paper, namely the factor A_L/A_S from MARTIN'S equation[3] $R_F = \dfrac{A_L}{A_L + \alpha A_S}$, since α can be obtained from the counter-current experiment (because here $\dfrac{A_L}{A_S} = 1$) and R_F from the paper chromatogram.

For Bi and Cd the values are 0.63 and 0.60 respectively. Two further values for A_L/A_S were also obtained from partition experiments with $ZnCl_2$ and $AsCl_3$ which were extracted in separating funnels from N HCl with butanol. The value of α so obtained depends on the concentration of Zn and As. If extrapolated to zero the value of A_L/A_S becomes 0.63 for Zn and 0.65 for As. These values are thus in agreement with the above values obtained with Cd and Bi. We suggest that for the calculation of partition coefficients from R_F values for Whatman No. 1 paper and butanol–N HCl, the value of 0.60–0.65 may be taken for A_L/A_S.

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We wish to thank the Faculty of Chemistry and Pharmacy of Montevideo for the fellowship that enabled us to carry out this work.

SUMMARY

The sequence of Bi–Cd in butanol-N HCl was found to vary with the degree of saturation of the paper. This can explain differences in results obtained in different laboratories. The factor A_L/A_S was determined for butanol-N HCl and Whatman No. 1 paper to be 0.60–0.65.

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SÉPARATION CHROMATOGRAPHIQUE SUR PAPIER DE QUELQUES ANIONS MINÉRAUX RENFERMANT DU SOUFRE ET LEUR IDENTIFICATION PAR SPECTROGRAPHIE INFRAROUGE

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INTRODUCTION

Nous avons donné[1] les premiers résultats de nos études sur la séparation rapide des ions sulfurique SO_4^{--}, hydrogénosulfurique SO_4H^-, pyrosulfurique $S_2O_7^{--}$, persulfurique $S_2O_8^{--}$, thiosulfurique $S_2O_3^{--}$, dithionique $S_2O_6^{--}$, trithionique $S_3O_6^{--}$, tétrathionique $S_4O_6^{--}$ et pentathionique $S_5O_6^{--}$ sur quelques μg en faisant varier les techniques déjà publiées (Pollard, McOmie et Jones[2], Scoffone et Carini[3]). Jusqu'ici nous avons opéré par chromatographie ascendante et unidimensionnelle, sur papier d'Arches No. 302, avec des durées d'ascension de 16 heures à température de 16 ± 0.5°.

Nous avons sélectionné, comme donnant des résultats acceptables et reproductibles, deux sortes de solvants, d'une part, le mélange *n*-butanol–eau (75/25% en volume), d'autre part, le mélange isopropanol–eau (75/25% en volume).

DISTINCTION ET REPÉRAGE CHIMIQUE

On réalise ces opérations à l'aide des réactifs suivants:

Solution aqueuse de chlorure de fer-III à environ 1% et solution de fer-III-cyanure de potassium également à 1% pour les thiosulfates,

Solution alcoolique de benzidine à 0.05% ou solution dans l'acide acétique dilué, ce qui donne, d'après une réaction bien connue, une coloration bleue avec les persulfates,

Solution alcoolique de benzidine, puis, aussitôt, une solution de permanganate de potassium environ *N*/100; les ions SO_4^{--}, SO_4H^-, $S_2O_7^{--}$, $S_2O_6^{--}$, $S_4O_6^{--}$ et $S_5O_6^{--}$ qui ne fournissaient pas de réaction assez sensible sur papier-filtre accusent alors la production d'une tache bleue sur fond brun; la limite de sensibilité observée est de 8 à 10 μg dans une goutte de 1/100 ml pour les sulfates et les bisulfates, les divers thionates et 4 à 5 μg pour les pyrosulfates. Remarquons que la goutte d'eau pure témoin permet d'observer entre benzidine et permanganate une coloration bleue très faible mais qui se trouve renforcée par la présence de l'un quelconque des ions cités plus haut. La sensibilité de cette réaction diminue aussi quand le sulfate, par exemple, a été mis au contact du solvant dans la cloche chromatographique.

DISTINCTION SPECTROCHIMIQUE

Nous avons donné il y a quelques années[4] le principe d'une analyse fine, quasi-instantanée, sur une goutte de 1/100 ml de solution aqueuse par spectrographie infrarouge. L'appareil utilisé est le spectromètre Perkin-Elmer 12 C équipé avec un prisme de chlorure de sodium. Les cations simples sont transparents dans la région choisie de 900–1500 cm^{-1} mais la plupart des anions minéraux y présentent une ou deux bandes généralement très fortes. Nous nous sommes servis, en particulier des nombreux spectres publiés depuis 1940 avec JEAN LECOMTE notamment dans le *Bulletin de la Société chimique de France* et relatifs aux groupements XO_2, XO_3, X_2O_5, XO_4, X_2O_7, etc. Les cuves sont faites avec deux plaques circulaires (ou carrées ou rectangulaires) de 2.4 cm de diamètre, en bromo-iodure de thallium emprisonnant la goutte de solution bien calibrée. Nous opérons donc avec une couche de 0.02 mm ne renfermant souvent qu'une fraction de microgramme de substance.

En réalité, la partie utile de la cuve qui couvre la fente d'entrée du spectromètre est extrêmement faible. En moyenne, la quantité de solution qui sert vraiment pour le phénomène est fonction de la largeur de cette fente qui n'est parfois que de 10 μ, soit un rectangle de 8 mm de haut sur 0.01 mm de large; on peut donc dire qu'il y a dans la cuve 1/5460 seulement de la surface qui sert et les nombres que nous donnons pour la limite de dilution doivent être divisés par ce nombre 5460. Si donc, la position d'une tache est décelée sur une bande de papier-filtre parallèle disposée dans la cloche et ayant subi le traitement de vaporisage, on découpe un carré de papier circonscrit à cette tache, on lave avec une goutte ou deux d'eau bidistillée et l'on enregistre un petit fragment de spectre entre des longueurs d'onde connues à l'avance et délimitant une zone d'absorption caractéristique et spécifique. L'opération complète: préparation d'une cuve avec l'eau bidistillée, inscription du spectre à vide, confection de la cuve avec la goutte de solution, enregistrement de la portion de spectre correspondant au corps cherché sur 20 cm de long au maximum, étalonnage des bandes tracées, identification, ne dure que 17 à 20 minutes et l'on conserve un document traduisant fidèlement l'état ionique actuel de la microgoutte de solution aqueuse. Pour des essais effectués en série, quatre minutes suffisent car il n'est pas nécessaire de tracer chaque fois le spectre à vide.

Bien entendu, la bande caractéristique étant inscrite, on peut l'utiliser pour faire une détermination quantitative si l'on a tracé à l'avance une droite d'étalonnage avec 5 ou 6 teneurs différentes de la même substance et si l'on admet la validité de la loi de Beer pour de si faibles teneurs, ce qui est le plus souvent justifié.

Les fréquences suivantes ont été observées avec les divers sels de sodium et de potassium.

Ion sulfate. On obtient une bande forte bien connue dite triple dégénérée pour 1103 cm^{-1} qui est encore visible pour une teneur de 50 μg dans la goutte de 0.01 ml; elle se retrouve dans le spectre de l'hydrogénosulfate et elle est déplacée à 1117 cm^{-1} chez les thiosulfates; on la signale aussi comme caractéristique de l'hydrolyse des thionates, de l'oxydation des sulfites, etc.

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Ion hydrogénosulfate. On trouve trois bandes fortes pour 1051, 1103 et 1200 cm^{-1}, la seconde étant commune, comme on le voit, aux sulfates; cependant, suivant les concentrations, la bande médiane peut être décalée jusqu'à 1111 cm^{-1}; la bande de 1051 cm^{-1} est ultime et permet de déceler 250 μg dans la goutte.

Ion pyrosulfate. Il présente deux bandes moyennes à 1050 et 1190 cm^{-1} et quelquefois une bande faible d'intensité variable à 1099 cm^{-1} due à l'hydrolyse; la limite de dilution est la même que dans le cas des sulfates.

Ion persulfate. Il présente deux bandes fortes pour 1048 et 1269 cm^{-1} ne disparaissant qu'au-dessous de 24 μg dans la goutte, celle de 1269 cm^{-1} restant toujours plus longtemps visible que l'autre; la bande des sulfates vient se placer entre les deux précédentes et il est facile de suivre sur le spectre l'évolution d'un persulfate en sulfate.

Ion thiosulfate. On observe la présence de deux bandes fortes pour 996 et 1117 cm^{-1}; la première donne pour limite de sensibilité 89 μg et la seconde 179 μg (comptés en thiosulfate de sodium pentahydraté) dans la goutte habituelle de 1/100 ml.

Ion dithionate. Il donne une bande moyenne à 991 cm^{-1} et une bande forte à 1233 cm^{-1}; limite 50 μg.

Ion trithionate. Il montre une bande très forte à 1099 cm^{-1} et une bande faible variable à 1014 cm^{-1} (sulfate); limite 50 μg.

Ion tétrathionate. On constate la présence d'une bande faible à 1015 cm^{-1} (sulfate) et d'une bande forte à 1221 cm^{-1}; limite 50 μg.

Ion pentathionate. Il présente une bande faible à 993 cm^{-1} et une bande variable en intensité à 1103 cm^{-1} (sulfate); limite 60 μg.

RÉSULTATS DE LA CHROMATOGRAPHIE

Nous avons opéré avec des solutions de concentrations comprises entre 0.25 et 1% afin d'obtenir des taches circulaires autant que possible. Nous avons mesuré la valeur de R_F dans le cas des sels alcalins.

Avec le solvant butanol–eau, on observe les résultats suivants pour les anions pris isolément:

Ions	SO_4^{--}	SO_4H^-	$S_2O_8^{--}$	$S_2O_7^{--}$	$S_2O_3^{--}$	$S_2O_6^{--}$	$S_3O_6^{--}$	$S_4O_6^{--}$	$S_5O_6^{--}$
R_F	0	0	0.36	0.57	0	0	0	0	0

On constate donc que seuls les anions $S_2O_8^{--}$ et $S_2O_7^{--}$ ont migré, ce qui les distingue de tous les autres; de plus avec le solvant suivant, les valeurs de R_F sont alternées; ce sont les persulfates qui montent le plus haut dans le papier; les spectres infrarouges, comme nous l'avons vu, sont aussi très différents.

Avec le dissolvant isopropanol–eau et en opérant toujours dans les mêmes conditions de dilution et de température, on observe que cette fois tous les anions ont migré. Les trithionates, les tétrathionates et les pentathionates donnent, en plus de leur tache propre, une tache ovale qui paraît être double et dont les valeurs de R_F correspondent bien à celles des sulfates et des thiosulfates pris séparément. Les autres anions sont bien distincts excepté ceux des pyrosulfates et dithionates dont les R_F

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sont les mêmes, mais, comme nous l'avons vu, il est facile de faire la distinction par le mélange butanol–eau ou par l'examen en infrarouge:

Ions	SO_4^{--}	SO_4H^-	$S_2O_8^{--}$	$S_2O_7^{--}$	$S_2O_3^{--}$	$S_2O_6^{--}$	$S_3O_6^{--}$	$S_4O_6^{--}$	$S_5O_6^{--}$
R_F	0.11	0.21	0.44	0.24–0.27	0.17	0.25	0.30–0.12	0.34–0.12	0.36–0.12

Nous avons ensuite étudié ces anions en les mélangeant deux par deux afin d'observer leur comportement dans le mélange et pour savoir si les valeurs de R_F se modifiaient (Tableau I).

TABLEAU I

Ions mélangés	*R_F observés*	*Ions identifiés*
SO_4^{--} et SO_4H^-	0.10 0.21	SO_4^{--} et SO_4H^-
SO_4^{--} et $S_2O_8^{--}$	0.10 0.21 0.44	SO_4^{--}, SO_4H^-, $S_2O_8^{--}$
SO_4H^- et $S_2O_8^{--}$	0.10 0.21 0.44	SO_4^{--}, SO_4H^-, $S_2O_8^{--}$
SO_4^{--} et $S_2O_7^{--}$	0.10 0.22	SO_4^{--}, $S_2O_7^{--}$
SO_4H^- et $S_2O_7^{--}$	0.11–0.12 0.24	SO_4^{--}, SO_4H^-, $S_2O_7^{--}$
$S_2O_7^{--}$ et $S_2O_8^{--}$	0.22–0.23 0.46–0.47	$S_2O_7^{--}$, $S_2O_8^{--}$
$S_2O_6^{--}$ et $S_3O_6^{--}$	0.12 0.22 0.31	SO_4^{--}, SO_4H^-, $S_2O_6^{--}$, $S_3O_6^{--}$
$S_3O_6^{--}$ et $S_5O_6^{--}$	0.12 0.31–0.35	SO_4^{--}, SO_4H^-, $S_3O_6^{--}$, $S_5O_6^{--}$

Cette sélection nous montre que la chromatographie décèle l'hydrolyse partielle. Dans presque tous les mélanges apparaît la tache ovale pour $R_F = 0.12$ et qui est due au mélange sulfate + hydrogénosulfate, résultat confirmé par spectrographie infrarouge.

RÉSUMÉ

A l'aide de deux solvants butanol–eau et isopropanol–eau, avec un nouveau réactif benzidine–permanganate et en s'aidant de la spectrographie d'absorption infrarouge réalisée sur une goutte de solution aqueuse, les auteurs étudient le comportement chromatographique d'un certain nombre d'anions dérivés du soufre, pris seuls ou deux par deux. La méthode se prête à une détermination quantitative réalisée sur quelques microgrammes.

SUMMARY

A study was made of the chromatographic behaviour of a number of anions derived from sulphur, solutions containing one anion or mixtures of two anions being examined. Use was made of the two solvents butanol–water and isopropanol–water, a new reagent benzidine–permanganate and infrared absorption spectrography carried out on a drop of aqueous solution. The method is suitable for quantitative determinations of microgram amounts.

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A SIMPLE PAPER-CHROMATOGRAPHIC METHOD FOR COMPARATIVE STUDIES OF ARGINASE IN ANIMAL TISSUES

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There are many methods for the determination of arginase. In most of them the urea formed is determined enzymically by the urease method or by means of some colour reaction. In our laboratory a simple chromatographic method was elaborated which in our hands gave reproducible results. The principle of this method is based upon incubation of arginine with tissue extract and spectrophotometric determination of the remaining arginine, after it has been separated by means of paper chromatography.

The preparation of arginase extract from animal tissues was performed according to GREENSTEIN *et al.*[1]. The homogenization of tissues was carried out in a glass homogenizer. The composition of the incubation medium was as follows:

arginase extract	1.0 ml
L-arginine·HCl, 2% soln.	1.0 ml
phosphate buffer, pH 8.0	0.4 ml

The incubation was carried out in tubes at + 38°, and the reaction stopped by addition of 0.1 ml of 2% acetic acid and by placing the tubes in boiling water for 10 min. The hot samples were filtered and cooled, and 25 μl were then spotted on Whatman No. 1 filter paper "for chromatography". Each sample was chromatographed in duplicate. In each series control spots of arginine, ornithine, and arginine + ornithine were developed.

TABLE I

CHANGES IN COLOUR INTENSITY OF EXTRACTED ARGININE SPOTS

12 spots of a standard solution of L-arginine·HCl were placed on Whatman No. 1 filter paper and a chromatogram was developed in the usual way. The spots were extracted at different times after the detection with ninhydrin. The optical density was read directly after elution.

Time between detection of spots and beginning of the extraction	*Optical density at $\lambda = 510$ mμ*	
	Series I	*Series II*
2 h	0.085	0.088
4 h	0.115	0.106
6 h	0.102	0.104
12 h	0.131	0.128
24 h	0.145	0.147
5 days	0.135	0.128

We used paper sheets which were 40 cm long and 20 cm high. Ascending chromatograms were developed during 5–6 hours in a solvent mixture of phenol saturated with water (7:3, v/v) in the presence of 5 ml of 2% NaCN and 5 ml of 0.1% NH_4OH. After running the chromatograms the paper was dried at room temperature for 24 hours, and the arginine spots were detected by dipping the paper in 0.25% solution of ninhydrin in acetone. Subsequently, the sheets were kept in the dark for 24 hours. During that time the spots reached their maximum intensity and stability (Table I). The arginine spots* were then cut out and the colour extracted with 8 ml of 75% ethanol containing 0.005% $CuSO_4$[2]. As a blank, a piece of paper in the neighbourhood of the arginine spots was extracted. The size of all the pieces of paper taken for extraction must be the same. We found that 45 min was sufficient time for extraction. This was done directly in spectrophotometer cuvettes (tubes, size 19 × 150 mm). During the extraction the tubes were kept in darkness. The optical density was read by means of a Coleman Junior spectrophotometer, and the amount of arginine was calculated from a standard curve (Fig. 1). The maximum absorption of the arginine spots is at $\lambda = 510$ mμ

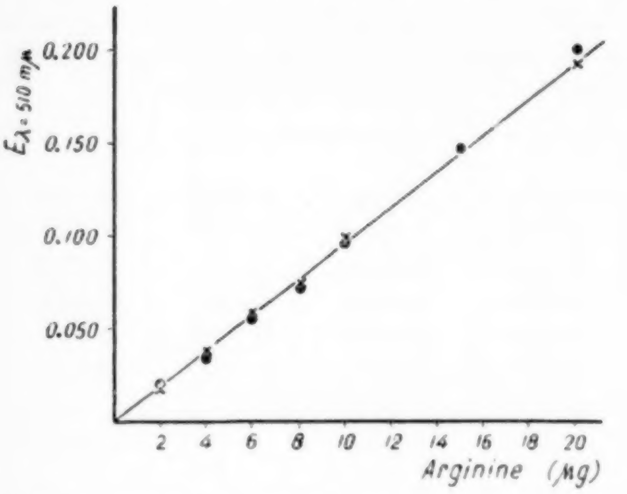

Fig. 1. Standard curve of arginine. Standard solutions of L-arginine·HCl were spotted on Whatman No. 1 filter paper, and a chromatogram developed by ascending technique (phenol–water = 7:3, + 0.1% NH_4OH, + 2% NaCN). The arginine spots were eluted 24 h after staining. The time of extraction = 45 min. The optical density was read directly after extraction.

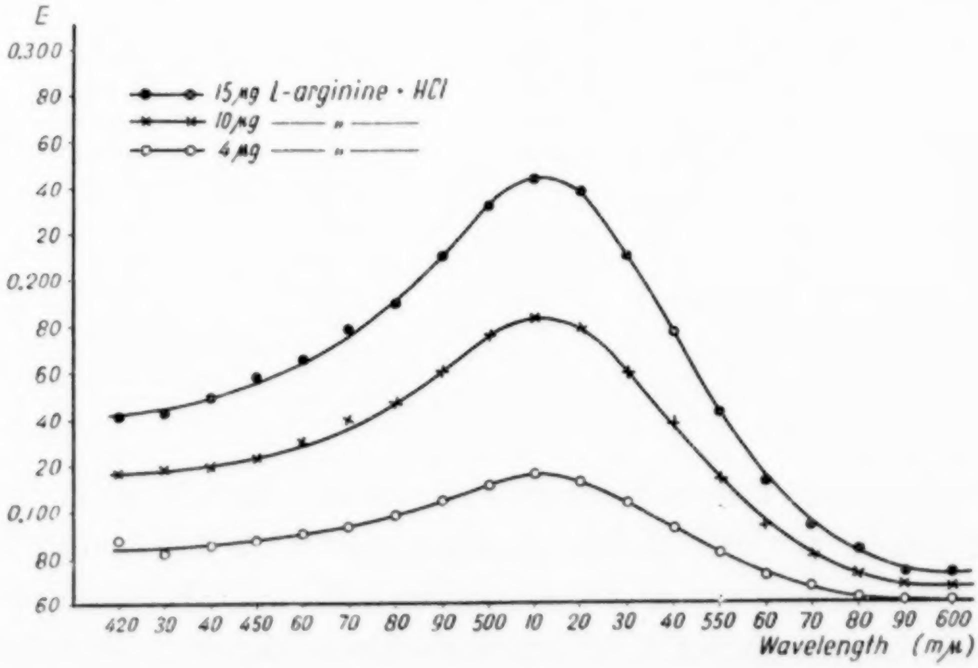

Fig. 2. Absorption spectrum of the arginine–ninhydrin colour. The arginine spots were eluted from the chromatogram with 75% ethanol containing 0.005% $CuSO_4$ 24 h after detection of the spots with ninhydrin. Eluting time = 45 min. The optical density relates to 8 ml of eluate and a light path of the cuvette of 16.0 mm.

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* On our chromatograms each sample gives four ninhydrin-positive spots; the arginine spot has the highest R_F value, the lower spot is ornithine, and the two remaining spots are unidentified products arising from the destruction of ornithine.

TABLE II

STABILITY OF THE ELUATES OF ARGININE SPOTS

The spots were extracted 24 h after staining of the chromatogram. Time of extraction = 45 min; the eluates were kept in darkness; the optical density was read at $\lambda = 510$ mμ.

Time after eluting (min)	*Sample No.*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
directly	0.040	0.041	0.080	0.080	0.089	0.090	0.170	0.172
5	0.039	0.040	0.080	0.080	0.089	0.090	0.170	0.172
10	0.039	0.040	0.080	0.080	0.090	0.090	0.171	0.173
15	0.039	0.040	0.080	0.080	0.089	0.090	0.171	0.173
20	0.039	0.040	0.079	0.079	0.089	0.090	0.170	0.172
30	0.039	0.041	0.078	0.079	0.088	0.088	0.170	0.171
60	0.039	0.041	0.078	0.079	0.089	0.087	—	—
120	0.039	0.041	0.078	0.079	0.089	0.088	—	—

TABLE III

RECOVERY OF ARGININE FROM THE CHROMATOGRAM

7 spots of 4, 10 and 15 μg of standard solution of L-arginine·HCl were spotted on Whatman No. 1 filter paper. Chromatograms were developed in the usual way. The spots were eluted 24 h after staining the chromatogram. The volume of the eluates = 8 ml. The optical density was read directly after eluting. The amount of arginine was estimated from the curve of standard spots developed simultaneously.

Sample No.	*Arginine recovered (μg)*		
	Arg. spotted 4.0 μg	*Arg. spotted 10.0 μg*	*Arg. spotted 15.0 μg*
1	4.0	9.7	14.7
2	3.7	9.9	15.0
3	3.8	9.9	15.3
4	4.0	9.7	15.3
5	3.6	10.0	15.3
6	4.6	10.0	14.6
7	4.1	9.9	14.7
Recovery (average)	3.98	9.88	14.98
Recovery (%)	99.5	98.8	99.9

(Fig. 2), and therefore this wavelength was used. As can be seen from Table II the extracted colour is quite stable over a period of 2 hours, and this stability is independent of the concentration of the arginine in the spot. Exposure of the extracts to light diminished the stability of the colour somewhat. The recovery of arginine spotted on the paper and developed by the technique of ascending chromatography was satisfactory (Table III). The recovery of arginine that had been added to diluted and immediately inactivated extract of rat liver was also good (Table IV).

The optical density of the extracted colour of arginine is proportional to the arginine concentration within a wide range (Fig. 1). However, some fluctuations in the inclination of the standard curve were observed. Therefore standard spots of

TABLE IV

RECOVERY OF ARGININE ADDED TO RAT LIVER EXTRACT

To 1.0 ml of liver extract, diluted 1:300, were added: 0.4 ml of phosphate buffer, pH 8.0, and 1.0 ml of standard solutions of L-arginine·HCl in such concentration as to make 4, 8 and 16 μg of arginine in 25 μl of the final volume. 0.1 ml of 2% acetic acid was added immediately to the mixture and the sample was boiled for 10 min. 25 μl of filtrate was spotted on Whatman No. 1 filter paper and the chromatogram was developed in the usual way.

Sample No.	*Arginine recovered (µg)*		
	Arg. spotted 4.0 µg	*Arg. spotted 8.0 µg*	*Arg. spotted 16.0 µg*
1	4.2	7.9	15.9
2	3.7	7.8	17.1
3	4.2	8.6	15.8
4	4.1	8.4	15.3
5	4.2	8.1	15.5
6	4.7	8.1	16.1
7	sample destroyed	8.0	16.3
Recovery (average)	4.18	8.13	16.0
Recovery (%)	104.5	101.6	100.0

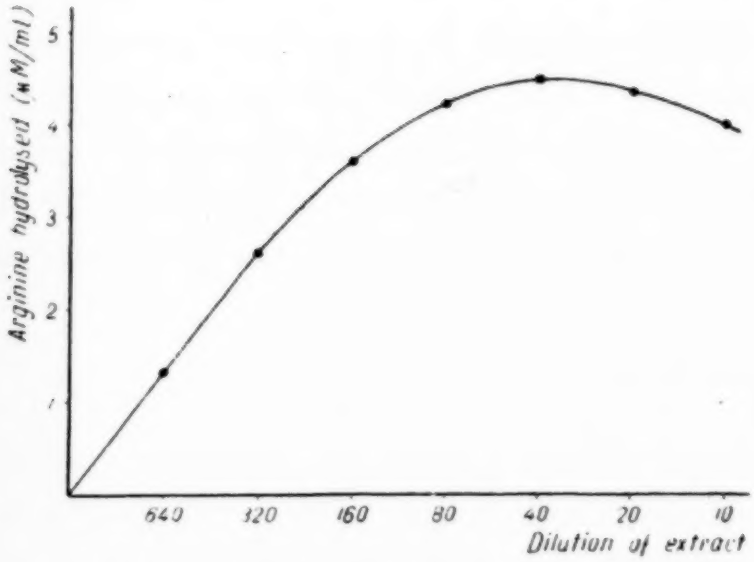

Fig. 3. The relationship between dilution of rat liver extract and degree of arginine hydrolysis. Incubation time = 30 min.

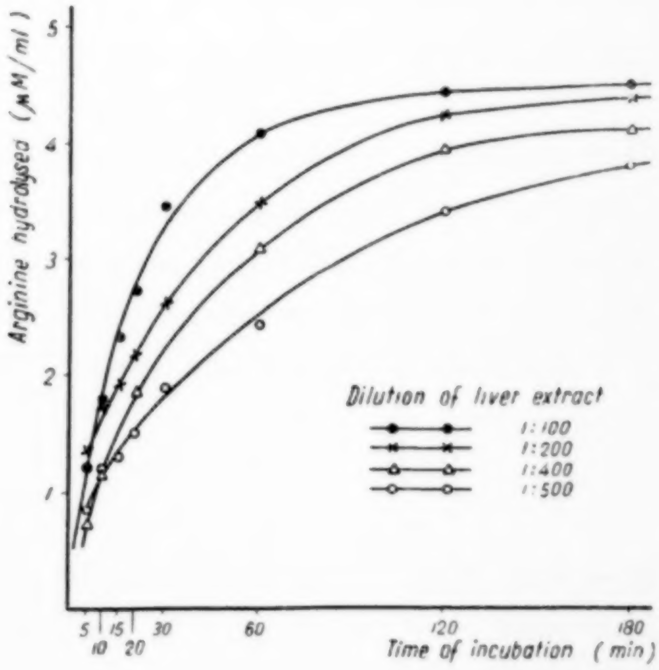

Fig. 4. The relationship between the degree of arginine hydrolysis and liver extract dilution, as well as incubation time.

arginine were developed simultaneously with each series of samples studied. Usually, 40–60 samples and one series of standard solutions (2, 4, 8, 15, 20 μg) of arginine were developed at the same time.

For the determination of arginase in the extracts of rat liver, an extract diluted 1:300 (w/v) with a solution of 0.5 M KCl + 0.03 N $NaHCO_3$ (1:1)[1] was used. In this range of dilution the amount of hydrolysed arginine is proportional to the degree of dilution of the extract (*i.e.* to the enzyme concentration) (Fig. 3). In Fig. 4 the

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relationship is shown between the degree of arginine hydrolysis and dilution of rat liver extract at different incubation times, as determined by our method.

SUMMARY

A simple paper-chromatographic method for arginase studies is described. In this method the arginine remaining after incubation with tissue extract is determined spectrophotometrically after it has been separated by paper chromatography.

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DETERMINATION OF N,N'-DIPHENYL-*p*-PHENYLENEDIAMINE (DPPD) IN MILK BY PAPER CHROMATOGRAPHY

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In previous studies from this laboratory, TEICHMAN *et al.*[1, 2] reported that feeding DPPD to lactating cows was effective in the prevention of copper-induced oxidized milk flavor. The presence of DPPD in the milk was confirmed qualitatively by the acetone–nitric acid method of PUDELKIEWICZ[3], a modification of the method originally proposed by BUNNELL[4] in which DPPD was quantitatively determined by the red color it produces in a concentrated nitric acid solution. This modification permitted quantitative DPPD determinations in chicken fat and eggs, whose extracts were not entirely soluble in concentrated nitric acid. However, quantitative estimations of DPPD were not possible in the studies of TEICHMAN *et al.*, because a portion of the non-saponifiable fraction (NSF) of milk was insoluble in the acetone–nitric acid mixture.

During the course of further experiments in this laboratory, concerned with determining the distribution of DPPD among the various milk fractions, the need arose for quantitative information on the concentration of DPPD present in milk. In view of the above-mentioned difficulties, the use of paper chromatography appeared promising as a possible means of separating DPPD from this insoluble material.

EXPERIMENTAL

Benzene solutions of purified DPPD* were chromatographed on 2" wide sheets of Whatman No. 1 filter paper. The solution was applied as a streak at the origin, and the chromatogram was allowed to develop overnight in the ascending direction, using 80% methanol as the developing solvent. After drying the paper was sprayed with concentrated nitric acid, and DPPD was visible as a red band migrating between R_F 0.75 and 0.80. Since the color produced by DPPD in concentrated nitric acid absorbs approximately three times as much light per unit of weight as the acetone–nitric acid color, concentrated nitric acid was utilized in the following determinations. For quantitative work, a band between approximately R_F 0.70 and 0.85 was cut from the chromatogram, 12 ml of concentrated HNO_3 was added to the paper (previously cut into small segments) in a glass stoppered tube, the tube shaken vigorously for

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* 99.5 + % DPPD, obtained from B. F. Goodrich Chemical Company, Cleveland, Ohio.

75 sec, the supernatant filtered through glass wool directly into an Evelyn tube, and the red color read, at the end of 2 min from the addition of the HNO_3, in an Evelyn colorimeter at 490 mμ. Concentrated nitric acid was used for the blank setting. The concentration of DPPD was determined from a standard curve obtained from known concentrations of DPPD in concentrated nitric acid covering the range of 5–25 γ DPPD per 12 ml.

The NSF from milk was obtained by lyophilization, extraction of the solids with ethyl ether, saponification of the ether extract with alcoholic KOH, and removal of the saponified material with water. The NSF was made up to a volume of 0.2 ml with benzene. Portions of this solution were then streaked on 2" wide filter paper sheets as described above.

RESULTS AND DISCUSSION

Chromatography of the NSF (consisting primarily of carotenoids, sterols, and phospholipids) from "blank" milk, revealed that no visible material migrated in the general area of DPPD. However, on treatment with concentrated nitric acid, a light brown band was observed in this area which absorbed light at 490 mμ in the colorimeter. Thus for subsequent determinations of DPPD in milk, a blank correction was required. All material migrating in the band between R_F 0.70 and 0.85 was found to be completely soluble in concentrated nitric acid. The quantitative nature of the proposed analysis was investigated by measuring recovery of DPPD at various stages in the procedure, and these results are given in Table I. Recovery of DPPD when chromatographed alone or in combination with the NSF of "blank" milk was of fairly high magnitude, 95.8% and 88.38% respectively. A mean recovery of 51.44% was obtained on the total method (DPPD added to milk prior to lyophilization) and indicated approximately 35% loss of DPPD in extraction of the NSF. However, as can be seen in Table I, the percentage recovery was rather consistent. The total method recovery, based on 4 samples carried through the entire procedure in duplicate, had a duplicate variance of 0.305 γ or 5.43% which indicated rather reasonable

TABLE I

RECOVERY OF DPPD

Treatment	*No. of determinations*	*Theoretical range of concentration* (γ)	*Mean recovery of DPPD* (%)
Chromatography of pure DPPD solutions	10	12.44 to 12.50	95.80 ± 1.29*
Pure DPPD solutions added to NSF fraction of "blank" milk prior to chromatography	4	6.0 to 12.5	88.38 ± 5.90
Total method, DPPD added to "blank" milk prior to lyophilization	8	5.0 to 6.25	51.44 ± 3.85

* Standard error of the mean, $s_{\bar{x}}$.

precision. BUNNELL[4] indicated that heat destruction of DPPD occurs, and this observation was confirmed in this laboratory. Heating on a steam bath for 15 min resulted in a 4 to 5% loss of DPPD, while saponfication of 4 samples of pure DPPD, extraction from the mixture with ether, and direct determination of DPPD after removal of the solvent gave a mean recovery of 81%. Thus, it was believed that the low recovery for the total method was due to heat and handling losses.

The use of acetylated paper for chromatographic separation of DPPD, as suggested by ZIJP[5] in studies on antioxidants in rubber, gave lower recoveries with pure DPPD solutions (85% versus 95%). Reversed-phase chromatograms (prepared with silicone or mineral oil), resulted in the extraction of oily droplets insoluble in concentrated nitric acid. For qualitative work, chromatography on partially acetylated filter paper using methanol–acetone–water (7:1:2) as a developing solvent allowed partial separation of DPPD and the "artifact" material present in milk. In this system DPPD had an R_F value of 0.30.

SUMMARY

A method for the determination of DPPD in milk by paper chromatography is reported. The chromatographic portion of the proposed procedure was found to be quantitative; however, factors in the extraction of DPPD from milk limit the total method to one of semi-quantitative nature. Percentage recoveries for the total method were reasonably consistent, and a high precision was noted.

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ZUR CHROMATOGRAPHISCHEN SPALTUNG VON RACEMISCHEN VERBINDUNGEN

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PROBLEMSTELLUNG

Lässt man durch eine Tswett'sche Adsorptionssäule, die mit einem optisch aktiven Adsorbens gefüllt ist, die Lösung eines Racemates durchgehen, so beobachtet man, wie eine Reihe von Autoren gezeigt hat[1], eine aktivierung der Lösung unter teilweiser Spaltung in die optischen Antipoden. Diese Spaltungen wurden durch die Annahme gedeutet, dass die Oberfläche des optisch aktiven Adsorbens eine selektive Adsorption für die beiden Antipoden aufweist, was zu einer Aktivierung der Racematlösungen führt[*]. In allen bisher untersuchten Fällen ist jedoch keine im präparativen Sinne ergiebige Spaltung erzielt worden.

In der vorliegenden Arbeit haben wir den Versuch unternommen, den Bereich der optisch aktiven Adsorbentien dadurch zu erweitern, dass wir ein optisch inaktives Adsorbens von grosser spezifischer Oberfläche, wie das Al_2O_3 für chromatographische Zwecke, mit einer dünnen Schicht von nur einigen Moleküllagen einer optisch aktiven Verbindung belegten, um es als Trennmittel in der chromatographischen Säule zu verwenden.

Durch dieses Verfahren, das wir zum ersten Male beim Versuch, ein racemisches, freies Radikal zu spalten, zur Anwendung brachten[5], wird ausser der grossen Ersparnis an optisch aktivem Material die erforderliche grosse Oberfläche durch das Aluminiumoxyd, über welchem die aktive Verbindung ausgebreitet ist, geschaffen. Durch Pulverisieren des kompakten, optisch aktiven Materials dagegen kann nicht leicht eine grosse specifische Oberfläche erreicht werden. Nur im Falle der Stärke, die von KREBS und Mitarbeitern erfolgreich zur Spaltung von Racematen herangezogen worden ist, herrschen bezüglich der spezifischen Oberfläche günstige Verhältnisse. Ein weiterer Vorteil der hier vorgeschlagenen Arbeitsweise besteht in der Möglichkeit, durch geeignete Wahl das zu spaltende Racemat stereochemisch an die gespreiteten, optisch aktiven Verbindungen anzupassen.

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[*] SCHWAB UND WAHL[2] haben mitgeteilt, dass sie bei der Wiederholung unserer Versuche, durch welche am Beispiel eines Cr-Komplexsalzes und optisch aktivem Quarz als Adsorptionsmittel, zum ersten Male die chromatographische Aktivierung einer racemischen Lösung demonstriert wurde, keine Drehung beobachten konnten. Bei der Durchsicht der dieser Mitteilung zugrundeliegenden Dissertation von WAHL[3] erkennt man jedoch, dass die genannten Autoren Bedingungen anwandten, unter welchen keine Drehung festgestellt werden kann. Die verwandte Menge des optisch aktiven Quarzpulvers, dessen Feinheitsgrad nicht angegeben wird, war nur ein Zehntel des von uns benutzten. Überdies haben SCHWAB UND WAHL das aktive Quarzpulver mit Salpetersäure vorbehandelt. Diese Prozedur verursacht eine leichte Peptisation des Quarzes, das sich nachträglich als amorphes SiO_2 an der Oberfläche der aktiven Kristalle niederschlägt und so die an und für sich geringe Selektivität der optisch aktiven, kristallinen Oberfläche auf Null herabdrückt. Bezüglich einer von zweiter Seite erfolgreichen Spaltung derselben Chromkomplexsalze an optisch aktivem Quarzpulver, siehe: SCHWEITZER UND TALBOTT[4].

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VERSUCHE

Die Versuche wurden in einer Säule von 2.5 cm lichter Weite und 1 m Höhe durchgeführt. Die Belegung des Al_2O_3 mit der optisch aktiven Substanz erfolgte dadurch, dass die in Lösung befindliche Verbindung mit der zur Bildung einer bimolekularen Schicht erforderlichen Menge Al_2O_3 zusammengebracht, das Lösungsmittel im Vakuum verdampft und die Mischung zwecks Ausbreitung der Verbindung auf der Aluminiumoxydoberfläche einige Stunden auf erhöhter Temperatur gehalten wurde. Mit diesem optisch aktiven Adsorbens wurde die Tswett'sche Säule gefüllt und durch diese die racemischen Lösungen hindurchgeleitet. Die Filtrate wurden in Fraktionen von *ca.* 25 ml nach Durchgang durch die Säule aufgefangen und polarimetriert. Nachdem das gesamte Lösungsvolumen zugegeben war, wurde mit einem geeigneten Lösungsmittel solange eluiert, bis nach Möglichkeit die gesamte Menge der zu spaltenden Verbindung aus der Säule wieder herausgewaschen war. Auch die Eluate wurden in Fraktionen von je 25 ml aufgefangen und polarimetriert.

Bei der Wahl der Lösungs- bzw. Elutionsmittel musste die Bedingung erfüllt sein, dass die zu spaltenden chemischen Verbindungen in den angewandten Lösungsmitteln mässig löslich, dagegen die als Belegung dienenden optisch aktiven Verbindungen, an deren Oberfläche die Spaltung erfolgen soll, unlöslich sind. Durch diese Forderung wird natürlich die Zahl der Stoffkombinationen eingeschränkt. Trotz der im blinden Versuch erwiesenen Unlöslickheit der Belegungen in den angewandten Lösungsmitteln, wanderten sie in einigen Fällen in geringen Mengen mit den Lösungsmitteln, ein Verhalten, welches wohl der Wanderung von anorganischen Salzen mit organischen Lösungsmitteln in der Papierchromatographie an die Seite zu stellen ist[6]. In solchen Fällen wurde das Eluat bis zur Trockene eingedampft und zwecks Polarimetrierung in einem Lösungsmittel aufgenommen, in welchem nur die mehr oder minder aktiven racemischen Verbindungen löslich waren. Nach der beschriebenen Methode wurde eine Reihe von Spaltungen erzielt, die an den folgenden Beispielen erläutert seien.

dl-Mandelsäure wurde durch selektive Adsorption an *d*-Weinsäure, die über Aluminiumoxyd in etwa mono- bis bimolekularer Schicht ausgebreitet war, bis zu einem Betrag von 13% in die Antipoden gespalten. Die spezifische Oberfläche des Aluminiumoxyds wurde nach der Impfmethode[7] zu 314 m^2/g ermittelt. Unter der Annahme, dass die *d*-Weinsäure auf der Aluminiumoxydoberfläche einen Flächenbedarf von 20 $Å^2$ besitzt, wurden 0.45 g *d*-Weinsäure auf 1 g Aluminiumoxyd ausgebreitet. Die Bedingung für die Spreitung einer Substanz über eine feste Oberfläche ist durch die Gleichung gegeben:

$$\gamma_{\text{fest}} - (\gamma_{\text{fest.fl.}} + \gamma_{\text{fl.}}) > 0$$

d.h. die spezifische freie Oberflächenenergie der festen Substanz soll grösser sein als

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die Summe der Grenzflächenspannung fest–flüssig und der Oberflächenspannung der zu spreitenden Flüssigkeit. Diese Bedingung ist wohl beim Al_2O_3 und den angewandten polaren organischen Verbindungen erfüllt.

Durch diese aktive Säulenfüllung liessen wir eine 7%ige *dl*-Mandelsäurelösung in einer Mischung von Aceton und leicht siedendem Petroläther (1:1) durchgehen.

TABELLE I

BEOBACHTETE DREHUNGEN AN FRAKTIONEN VON 25 ml

Proben No.	*Drehung*	*Proben No.*	*Drehung*
1	— 0.25°	8	+ 1.32°
2	— 0.75°	9	+ 1.03°
3	— 0.41°	10	+ 0.88°
4	— 0.52°	11	+ 0.83°
5	— 1.20°	12	+ 0.70°
6	— 0.25°	13	+ 0.69°
7	+ 0.65°		

Nachdem 200 ml der Lösung hindurchgegangen waren, wurde mit reinem Aceton eluiert. Tabelle I enthält die bei den einzelnen Fraktionen beobachteten Drehungen für die Wellenlänge $\lambda = 5200$ Å. Die Genauigkeit der Ablesung schwankte zwischen ± 0.01° und ± 0.02°.

Aus dem Verlauf der beobachteten Drehungen muss man schliessen, dass die *l*-Mandelsäure stärker an die *d*-Weinsäure adsorbiert wird als ihre Antipode. Die Fraktionen mit gleichem Drehungssinn wurden vereinigt, zur Trockene eingedampft, mit absolutem Benzol extrahiert, um eventuell mitgegangene *d*-Weinsäure auszuschliessen, der Extrakt wieder zur Trockene eingedampft und zur Bestimmung der spezifischen Drehung in Wasser aufgenommen. Es wurden spezifische Drehungen von $[\alpha] = +13.5°$ bzw. $[\alpha] = -12.1°$ gemessen. Da die spezifische Drehung der *d*-Mandelsäure + 193.4° beträgt, war die relative Anreicherung des Racemates für jede der beiden Antipoden etwa 6.5% womit eine 13%ige Spaltung der angewandten Mandelsäure durch selektive Adsorption erreicht wurde. Ein Versuch mit *dl*-Mandelsäure in einer Chloroform–Acetonmischung (3:1) als Lösungsmittel führt im Endergebnis zu der gleichen 13%igen Spaltung der Mandelsäure.

Als zweites Beispiel sei die Aktivierung einer *dl*-Menthollösung durch Adsorption an Aluminiumoxyd, welches mit L-glutaminsaurem-Natrium, $HOOC–CH_2–CH_2–CH(NH_2)–COONa$ belegt war. Die Lösungen zeigten nach dem Eluieren mit Petroläther die Drehwerte von + 0.25° bzw. — 0.27°, was, berechnet auf die angewandte Mentholmenge, etwa einer 1%igen Spaltung entspricht. In analoger Weise ist mit der anderen Stoffkombination verfahren worden.

In Tabelle II sind die Versuche zusammengestellt, welche zu einer teilweisen Spaltung der Racematlösung führten. Tabelle III enthält die Substanzkombinationen, bei welchen keine nachweisbare Spaltung beobachtet werden konnte. In der letzten Rubrik ist der wahrscheinliche Grund für das Ausbleiben der Aktivierung für den jeweiligen Versuch angeführt.

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TABELLE II

SUBSTANZKOMBINATIONEN, DIE ZU EINER RACEMATTRENNUNG FÜHRTEN

Adsorbens Al_2O_3 belegt mit:	*g aktive Subst. pro g Al_2O_3*	*Racemat*	*Lösungsmittel*	*Elut. mittel*	*Max. abgel. Drehung*	*Spez. Drehung*	*% Spaltung*
d-Weinsäure	0.45	*dl*-Mandelsäure 7.5% Lösg.	Petroläther + Aceton (1:1)	Aceton	+ 1.45° — 1.31°	+ 13.5°	13
d-Weinsäure	0.45	*dl*-Mandelsäure 4% Lösg.	Chloroform + Aceton (3.5:1)	Chloroform + Aceton (4:1)	+ 1.29° — 0.49°	+ 12.5° — 13.0°	13
d-Weinsäure	0.40	*dl*-Mandelsäure 8% Lösg.	Petroläther + Aceton (3:2)	Petroläther + Aceton (2:1)	+ 0.37° — 3.66°	+ 8.5° — 12.0°	10.5
D-Glukose	0.42	*dl*-Mandelsäure 6% Lösg.	Petroläther + Äther (0.3:1)	Petroläther + Äther	+ 0.16° — 0.39°	+ 5.7° — 2.2°	9.0
d-Weinsäure	0.43	*dl*-Mandelsäure-methylester 15% Lösg.	Benzol	Benzol	+ 0.82° — 0.77°	+ 1.2° — 1.5°	1.4
d-Weinsäure	0.43	*dl*-*α*-Aminophenyl-essigsäuremethyl-ester 10% Lösg.	Benzol + Ligroin (1:2)	Benzol + Lig. Benzol Äther	— 1.09°	— 10.9°	6.0
L-Glutaminsaures Natrium	0.35	*dl*-Menthol 3% Lösg.	Petroläther	Petroläther	+ 0.25° — 0.27°	+ 0.5° — 0.54°	1.0
D-Laktose	0.39	*dl*-Dinitrodi-phensäuredime-thylester 9% Lösg.	Petroläther + Benzol (1:9)	Petroläther + Benzol (1:9)	+ 0.20° — 0.10°		
d-Fenchon		*dl*-*α*-Aminophenyl-essigsäure	Wasser	Wasser	+ 0.12° — 1.108°		8.0

TABELLE III

SUBSTANZKOMBINATIONEN, DIE ZU KEINER RACEMATTRENNUNG FÜHRTEN

Adsorbens Al_2O_3 belegt mit:	*g akt. Subst. pro g Al_2O_3*	*Racemat*	*Lösungsmittel*	*Elut. mittel*	*Vermuteter Grund für die Nichtaktivierung*
l-Menthol	0.35	*dl*-Mandelsäure 14% Lösg.	Wasser	Wasser	4/5 der Mandelsäure waren nicht mehr zu eluieren. Ungenügende Spreitung des Menthols auf Al_2O_3.
L-Glutaminsaures Natrium	0.37	*dl*-Menthol 17% Lösg.	Aceton + Wasser (3:1)	Wasser + Aceton (1:3)	Keine Adsorption. Wasserhülle um Glutaminsaures Natrium verhindert Wechselwirkung zwischen Adsorbens und Adsorbendum.
d-Weinsäure	0.58	*dl*-*α*-Naphthylpropionsäure 6% Lösg.	Petroläther	Petroläther	Adsorption fand statt. Keine Selektivität.
d-Weinsäure	0.54	*dl*-Tropasäure 8% Lösg.	Petroläther + Aceton (3:2)	Petroläther + Aceton	Adsorption fand statt. Keine Selektivität.
d-Weinsäure	0.45	*dl*-Campher 17% Lösg.	Petroläther	Petroläther	Keine Adsorption wegen grosser Löslichkeit in allen Lösungsmitteln.
D-Laktose	0.39	*dl*-Dinitrodiphensäure 6.8% Lösg.	Petroläther + Aceton (4:1)	Petroläther + Aceton, Aceton, Methanol	Nebenreaktion wahrscheinlich Veresterung.
L-Glutaminsaures Natrium	0.37	*dl*-Tropasäure 2.3% Lösg.	Benzol + Äther (1:2)	Benzol, Äther, Alkohol	Zu starke Adsorption. Keine Elution möglich.
D-Glukose		*dl*-Phenylbenzylmethylcarbinol	Petroläther	Petroläther	Keine Adsorption.
d-Weinsäure		*dl*-Phenylbenzyl methylcarbinol	Petroläther	Petroläther	Keine Adsorption.
d-Weinsäure		*dl*-Dibrenztraubensäurepentaerythrit		Petroläther	Starke Adsorption, keine Selektivität.

Bereits die hier angeführten Beispiele lassen einen Zusammenhang zwischen dem sterischen Bau der Racemate und den optisch aktiven Adsorbentien erkennen. Eine eingehende Diskussion möchten wir jedoch verschieben, bis ein umfangreicheres Material vorliegt.

DANK

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ZUSAMMENFASSUNG

Es ist experimentell an Hand von 9 aus insgesamt 19 versuchten Fällen gezeigt worden, dass eine Spaltung von Racematen in die optischen Antipoden durch selektive Adsorption an einem optisch inaktiven Adsorbens, wie Aluminiumoxyd, über welchem jedoch eine optisch aktive Substanz in Schichten von wenigen Moleküllagen ausgebreitet ist, möglich ist. Die vorgeschlagene experimentelle Methode erlaubt durch Anpassung der optisch aktiven Verbindung an das zu spaltende Racemat eine grössere Variation der Spaltung gelangenden Stoffe.

SUMMARY

Experiments, which were successful in 9 out of 19 cases, have shown that it is possible to resolve racemates into the optical antipodes by selective adsorption on an optically inactive adsorbent (such as aluminium oxide) coated with an optically active substance in layers a few molecules in thickness. By adapting the optically active substance to the racemate it is possible to resolve a fairly large variety of racemates with the proposed experimental method.

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THE CHROMATOGRAPHY OF POLYCYCLIC AROMATIC HYDROCARBONS ON ACETYLATED PAPER

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The technique of paper chromatography has been extensively used in the analysis of complex mixtures of compounds containing polar groups; but until recently, little work has been reported on the chromatography of relatively non-polar compounds. Such substances tend to move with the solvent front under conditions suitable for the separation of polar compounds, so that some modification of technique is necessary. MICHEEL[1] has successfully used partially acetylated paper for the separation of a number of hydrophobic compounds, and several authors have described the separation of polycyclic aromatic compounds on papers impregnated with vaseline, alumina, or propylene glycol[2], with N,N-dimethylformamide[3] and also on partially acetylated paper[2,4]. The results have been encouraging, but have not been of great analytical use owing to the difficulty of obtaining reproducible results. Moreover there was extensive overlapping of spots in the developed chromatograms, even in the few compounds studied. A satisfactory procedure for separating many polycyclic aromatic compounds by chromatography on acetylated paper, and for identifying the components of complex mixtures of polycyclic hydrocarbons by ultraviolet absorption spectra is now described.

Several methods of acetylating paper for chromatography have appeared in the literature[5,6,7,8], but it has been found that these are not invariably satisfactory. In general, the papers produced by these methods are not sufficiently acetylated to give good separations of polycyclic compounds. However, the procedure given in the experimental section has been found to give excellent papers, with reasonably reproducible properties, and high mechanical strength. Less highly acetylated papers prepared by using a smaller concentration of sulphuric acid catalyst proved moderately successful, giving somewhat smaller separations. Several solvent systems have been investigated, and the greatest resolution was obtained with a mixture of ethanol, toluene and water (17:4:1, v/v), and only slightly less with the system ethanol–benzene–water (12:6:1, v/v). However, extensive tailing occurred with the latter under certain circumstances.

The compounds were introduced onto the paper in chloroform or benzene solution by the usual spotting technique, and the papers developed by the ascent method for periods of 8 to 16 hours, depending on the solvent system used. During this time the solvent ascended to a height of 35 to 40 cm from the base line. (The descent method of development was found to accentuate the irregularities in the acetylated paper, and great care had to be exercised in the acetylation process before satisfactory results could be obtained.) Greatly increased separations between compounds with relatively low R_F values were obtained by developing the papers by the descent method and allowing the solvent front to overrun the end of the paper; but the technique was not invariably successful for the reason mentioned.

Most of the polycyclic compounds listed in Table I are strongly fluorescent and their position on the paper was determined simply by viewing under ultraviolet light. (The nature of the fluorescence is sometimes useful in distinguishing between compounds of approximately the same R_F value.) A method of locating non-fluorescent compounds, such as fluorene, phenanthrene, and triphenylene, by a "photoprint technique" has been described[4], but it is simpler to locate these compounds by spraying with a 2% solution of tetrachlorophthalic anhydride in a mixture of acetone and chlorobenzene (10:1, v/v), and again viewing the papers under ultraviolet light.

TABLE I

SEPARATION ON HIGHLY ACETYLATED PAPER

Solvent: Ethanol–toluene–water (17:4:1, v/v), 35 cm height of ascent.

Compound	R_F	*Fluorescence*
Acenaphthylene	0.576	none
Anthracene	0.494	dull violet
Anthanthrene	0.163	deep blue
Anthracenoanthracene	0.040	blue-violet
Azulene	0.700	—
3:4-Benzopyrene	0.093	violet
3:4-Benzofluoranthene	0.209	bright blue
2:3-Benzofluorene	0.539	none
1:2-Benzanthracene	0.396	violet
3:4-Benzophenanthrene	0.483	violet
1-Methyl-3:4-benzophenanthrene	0.546	violet
2-Methyl-3:4-benzophenanthrene	0.599	violet
3-Methyl-3:4-benzophenanthrene	0.506	violet
4-Methyl-3:4-benzophenanthrene	0.329	violet
5-Methyl-3:4-benzophenanthrene	0.509	violet
6-Methyl-3:4-benzophenanthrene	0.426	violet
Chrysene	0.206	none
20-Methylcholanthrene	0.281	blue-violet
Coronene	0.410	violet
1:2:5:6-Dibenzanthracene	0.246	violet
3:4:9:10-Dibenzopyrene	0.210	blue
2:3:10:11-Dibenzoperylene	0.042	dull blue
9,10-Diphenylanthracene	0.675	bright blue
1,1′-Dinaphthyl	0.539	dull violet
2,2′-Dinaphthyl	0.359	dull violet
9,9′-Diphenanthryl	0.531	none
Fluoranthene	0.441	light blue
Fluorene	0.401	none
Perylene	0.351	blue
Phenanthrene	0.446	none
Pyrene	0.471	violet
Picene	0.030	none
Triphenylene	0.501	none
Violanthrene	0.023	yellow

Under these conditions all the non-fluorescent compounds examined, with the exception of naphthalene, gave bright yellow or orange spots. Many weakly fluorescent compounds, such as the dinaphthyls, were found to be easily located by this method.

A summary of the R_F values obtained for a number of compounds is given in Tables I and II. The values given in Table I were obtained with papers acetylated with a mixture containing 0.6 g of concentrated sulphuric acid as catalyst, while those in Table II were obtained with papers of lower acetyl content, using 0.48 g of sulphuric acid as catalyst. The R_F values listed are intended only to give the order in which the compounds appeared on the paper, and the magnitude of the separations which may be expected. The actual R_F values obtained varied considerably from one batch of papers to another, but the order of the compounds appeared to be invariant under the experimental conditions. A comparison of the values given in the two tables shows that although the ratio of R_F values obtained for the papers of lower acetyl content is less than that for the more highly acetylated papers, the actual separations obtained for compounds with an R_F lower than that of 3:4-benzopyrene are greater, because of the overall increase in R_F values. For compounds with an R_F greater than that of 3:4-benzopyrene the separations obtained on the more highly acetylated papers are considerably greater.

TABLE II

SEPARATIONS ON PARTIALLY ACETYLATED PAPER

Solvent: Ethanol–toluene–water (17:4:1, v/v), 35 cm height of ascent.

Compound	R_F	*Fluorescence*
3:4-Benzopyrene	0.310	violet
Perylene	0.540	blue
Anthanthrene	0.360	deep blue
Anthracenoanthracene	0.070	blue-violet
2:3:10:11-Dibenzoperylene	0.160	dull blue
Violanthrene	0.050	yellow

The separations obtained by this method are appreciably better than those previously reported; but it is obvious that the technique in this form is not suitable for the analysis of complex mixtures of polycyclic hydrocarbons. The values recorded for the six isomeric methyl-3:4-benzophenanthrenes, for example, show that an analysis based only on comparison of R_F values could be grossly misleading. However, it is possible to identify the compounds present far more rigorously by cutting the spots out of the developed chromatograms, extracting with ether, and determining the ultraviolet absorption spectra of the extracts in a suitable solvent. It was usually found necessary to combine the extracts from several chromatograms to obtain sufficiently intense spectra, and it has been found that the spectra determined in this way reproduce those recorded in the literature both in the position of the bands and in their relative intensities. The same technique was also applied to the determination of the fluorescence spectra; but the characterisation of the compounds by this method must necessarily be less exact in view of the small number of spectra recorded. Non-fluorescent compounds were located by spraying a strip cut from the chromatogram, and then extracting the untreated paper in the manner described.

This technique is obviously applicable only when relatively clean separations can be obtained on the papers. Fortunately it has been found that most of the polycyclic compounds investigated can be separated by first effecting a preliminary separation by distillation, or by chromatography on alumina, or both, and then separating the components of the fractions obtained by the chromatographic techniques now described. In particularly difficult cases the spots can be cut out of the developed chromatograms and again chromatographed on acetylated paper, using an extended development time to obtain greater resolution; but this was seldom found to be necessary.

The technique has also been made semi-quantitative by using one of the methods of comparing the area and intensity of the spots with suitable standards, or better, by determining the concentration from the observed optical densities and the recorded extinction coefficients of the compounds. In those cases where all the components of the mixture can be identified, the relative concentrations can be determined with reasonable precision, but if this is not the case it is still possible to obtain a reasonably accurate estimate of the amount present by applying a known weight of the mixture to the chromatogram and using a standardised isolation procedure. It is necessary to determine the optical density at several points of the spectrum and to average the results to minimise the effect of background radiation.

This technique has been used extensively in these laboratories for the identification of the components of complex mixtures of polycyclic aromatic hydrocarbons obtained in the pyrolysis of some hydrocarbons, and its further application to the problem of atmospheric pollution is obvious. A similar technique has been found to be of use in the identification of a number of substituted indoles present in a reaction mixture, and also to be applicable to certain steroids.

EXPERIMENTAL

Acetylation technique

Ten strips of Whatman No. 1 chromatography paper 14 cm wide and 50 cm long were rolled together and placed in a wide-mouthed Erlenmeyer flask. Glass spacing rods were inserted into the roll at intervals to ensure even acetylation and the papers covered with an acetylating mixture comprising 800 ml of acetic anhydride, 1,000 ml of thiophen-free benzene and 0.6 g of concentrated sulphuric acid. The mixture was then heated under reflux at 60 to 65° for six hours with occasional shaking. After this time the acetylating mixture was poured off and the flask filled with absolute ethanol and allowed to stand overnight. The ethanol was poured off and the papers washed several times with distilled water and air-dried.

The nature of the paper obtained was found to depend on the purity of the benzene used and on the ratio of sulphuric acid to acetic anhydride. The benzene used must be free from sulphur compounds if reproducible results are to be obtained. The purity of the acetic anhydride did not appear to be of great importance, provided that the concentration of acetic acid present did not exceed 5%. Larger amounts of sulphuric acid gave a more highly acetylated paper, but this was found to possess no great superiority over the papers obtained by the technique described.

Solvent systems

The following solvent systems proved satisfactory:

Ethanol–toluene–water	17:4:1 v/v
Methanol–toluene–water	12:6:1 v/v
Methanol–toluene–water	10:1:1 v/v
Ethanol–benzene–water	12:6:1 v/v
Methanol–benzene–water	12:6:1 v/v
Methanol–ether–water	4:4:1 v/v

The solvent system used depended on the nature of the paper and the result desired. The solvents containing methanol were suitable for papers of a relatively low acetyl content, and those containing ethanol for papers with a high acetyl content. Mixtures of methanol and ethanol with light petroleum "tailed" badly. Other more exotic systems which have been proposed, were not as efficient as those recorded.

For the determination of the ultraviolet absorption spectra the preferred developing solvent is methanol–ether–water (4:4:1, v/v). Some loss of spectral fine structure occurred with the other solvent systems unless special precautions were taken to ensure complete removal of traces of benzene or toluene from the developed chromatograms.

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SUMMARY

A satisfactory technique for the separation of polycyclic aromatic compounds by chromatography on acetylated paper, and for identifying the components of complex mixtures, has been described.

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A CHROMATOGRAPHIC STUDY OF SOME OXALATE COMPLEXES

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The R_F value of a metal ion is known to depend on several factors, *viz.*, the concentration of the solution, the nature of the solvent employed, the presence of other ions, etc. Especially the presence of substances capable of yielding complex ions with the metal affects the diffusion of the ion through filter paper. MILLER and co-workers[1] observed the changes in the R_F value of certain metal ions on adding ammonia to the solvent and studied the effect of complexation of the metal ion with ammonia chromatographically. LEDERER[2] studied some cobalt ammine complexes by chromatography using solvents saturated with acid for developing the chromatograms.

In a recent communication SINGH AND DEY[3] studied the movement of metal ammines through filter paper strips using solutions containing a fixed quantity of the metal ion with varying concentrations of ammonia. In another paper[4] they investigated the behaviour of the complex cupri-oxalate ion by chromatography, using different proportions of cupric and oxalate ions. They extended the work to citrate[5] and tartrate[6] complexes of several metals as well. They noted that previous workers, such as POLLARD *et al.*[7,8] and LEDERER, studied the complexing property either by adding the complexing agent to the solvent or by impregnating the filter paper with the reagent. Hence their experiments create conditions where only the action of an excess of the complexing agent can be seen. The dependence of diffusion on the variations in the concentration of the complexing agent has not received much attention. Hence in this paper we have studied the diffusion of oxalate complexes of Ni^{++}, Co^{++}, Fe^{++} and Fe^{+++} in the presence of varying concentrations of oxalate ion.

EXPERIMENTAL

Standard solutions of 0.1 M $NiSO_4$; $CoSO_4$; $Fe_2(SO_4)_3$; and $(NH_4)_2SO_4 \cdot FeSO_4$ were prepared using BDH reagent grade chemicals. A standard solution of potassium oxalate was also prepared and standardised as usual. Mixtures containing a constant volume of the metal solution with varying proportions of oxalate were prepared. The ascending filter paper strip method was used as described by GAGE, DOUGLASS AND WENDER[9]. 50% ethanol was used as solvent. The filter paper was Whatman No. 1. Experiments were conducted at a room temperature of 30° and the time allowed was 90 minutes. The experimental results are given below.

Nickel oxalate complex

In the beginning precipitation of the oxalate occurred in the systems and when about 7 equivalents of oxalate were added total redissolution of the precipitate took place.

References p. 98.

TABLE I

Final concentration of $NiSO_4$, 0.05 *M*

	Ratio $Ni^{+2}:C_2O_4^{-2}$	R_F value
1	1:0	0.56
2	1:0.4	0.53
3	1:0.8	0.52
4	1:1.2	0.48
5	1:1.6	0.47
6	1:2.0	0.47
7	1:2.4	0
8	1:2.8	0
9	1:3.2	0.41
10	1:3.6	0.43
11	1:4.0	0.43
12	1:5.2	0.44
13	1:6.0	0.44
14	1:6.8	0.44
15	1:8.0	0.44
16	1:10.0	0.34
17	1:12.0	0.34
18	1:16.0	0.33
19	1:20.0	0.33

TABLE II

Final concentration of $NiSO_4$, 0.025 *M*

	Ratio $Ni^{+2}:C_2O_4^{-2}$	R_F value
1	1:0	0.53
2	1:0.8	0.50
3	1:1.6	0.50
4	1:2.4	0
5	1:3.2	0.34
6	1:4.0	0.48
7	1:4.8	0.48
8	1:5.6	0.48
9	1:6.4	0.45
10	1:7.2	0.45
11	1:8.0	0.45
12	1:10.4	0.40
13	1:12.0	0.40
14	1:13.6	0.40
15	1:16.0	0.34
16	1:20.0	0.32
17	1:24.0	0.32
18	1:32.0	0.32
19	1:40.0	0.32

TABLE III

Final concentration of $CoSO_4$, 0.025 *M*

	Ratio $Co^{+2}:C_2O_4^{-2}$	R_F value
1	1:0	0.72
2	1:0.4	0.69
3	1:0.8	0.66
4	1:1.2	0.13
5	1:1.6	0.57
6	1:2.0	0.58
7	1:2.4	0.50
8	1:2.8	0.49
9	1:3.2	0.49
10	1:3.6	0.48
11	1:4.0	0.42
12	1:5.2	0.42
13	1:6.0	0.41
14	1:6.8	0.41
15	1:8.0	0.39
16	1:10.0	0.39
17	1:12.0	0.39
18	1:16.0	0.38
19	1:20.0	0.38

TABLE IV

Final concentration of $CoSO_4$, 0.0125 *M*

	Ratio $Co^{+2}:C_2O_4^{-2}$	R_F value
1	1:0	0.55
2	1:0.8	0.34
3	1:1.6	0.31
4	1:2.4	0.51
5	1:3.2	0.51
6	1:4.0	0.52
7	1:4.8	0.53
8	1:5.6	0.49
9	1:6.4	0.47
10	1:7.2	0.47
11	1:8.0	0.46
12	1:10.4	0.44
13	1:12.0	0.43
14	1:13.6	0.42
15	1:16.0	0.42
16	1:20.0	0.42
17	1:24.0	0.41
18	1:32.0	0.41
19	1:40.0	0.40

In every case there was tailing in the chromatograms. From Tables I and II it can be seen that on adding oxalate to the metal, the R_F value decreases at first and then begins to increase as redissolution of the precipitate occurs and finally has a tendency to diminish again. The R_F value becomes zero with 2.4 to 2.8 equivalents of oxalate. The observations also show that the R_F values are less when dilute solutions of the metal are employed. The chromatograms were developed with dimethylglyoxime solution.

TABLE V

Final concentration of $Fe_2(SO_4)_3$, 0.05 M

	Ratio $Fe^{+3} : C_2O_4^{-2}$	R_F *value*
1	1:0	0.82
2	1:0.4	0.82
3	1:0.8	0.82
4	1:1.2	0.83
5	1:1.6	0.86
6	1:2.0	0.86
7	1:2.4	0.86
8	1:2.8	0.86
9	1:3.2	0.87
10	1:3.6	0.87
11	1:4.0	0.87
12	1:5.2	0.85
13	1:6.0	0.85
14	1:6.8	0.85
15	1:8.0	0.84
16	1:10.0	0.84
17	1:12.0	0.83
18	1:16.0	0.82
19	1:20.0	0.80

TABLE VI

Final concentration of $Fe_2(SO_4)_3$, 0.025 M

	Ratio $Fe^{+3} : C_2O_4^{-2}$	R_F *value*
1	1:0	0.77
2	1:0.8	0.77
3	1:1.6	0.77
4	1:2.4	0.78
5	1:3.2	0.83
6	1:4.0	0.82
7	1:4.8	0.82
8	1:5.6	0.81
9	1:6.4	0.81
10	1:7.2	0.80
11	1:8.0	0.80
12	1:10.4	0.80
13	1:12.0	0.80
14	1:13.6	0.79
15	1:16.0	0.79
16	1:20.0	0.79
17	1:24.0	0.79
18	1:32.0	0.79
19	1:40.0	0.79

TABLE VII

Final concentration of ferrous ammonium sulphate, 0.05 M

	Ratio $Fe^{+2} : C_2O_4^{-2}$	R_F *value*
1	1:0	0.79
2	1:0.4	0.69
3	1:0.8	0.67
4	1:1.2	0.67
5	1:1.6	0.65
6	1:2.0	0.65
7	1:2.4	0.42
8	1:2.8	0.52
9	1:3.2	0.72
10	1:3.6	0.74
11	1:4.0	0.76
12	1:5.2	0.76
13	1:6.0	0.77
14	1:6.8	0.78
15	1:8.0	0.78
16	1:10.0	0.78
17	1:12.0	0.78
18	1:16.0	0.78
19	1:20.0	0.78

TABLE VIII

Final concentration of ferrous ammonium sulphate, 0.025 M

	Ratio $Fe^{+2} : C_2O_4^{-2}$	R_F *value*
1	1:0	0.69
2	1:0.8	0.56
3	1:1.6	0.56
4	1:2.4	0.34
5	1:3.2	0.38
6	1:4.0	0.64
7	1:4.8	0.74
8	1:5.6	0.74
9	1:6.4	0.76
10	1:7.2	0.77
11	1:8.0	0.77
12	1:10.4	0.78
13	1:12.0	0.78
14	1:13.6	0.78
15	1:16.0	0.78
16	1:20.0	0.77
17	1:24.0	0.77
18	1:32.0	0.77
19	1:40.0	0.76

Cobalt oxalate complex

Precipitation of the oxalate occurs in all cases, until 8 equivalents of oxalate have been added, when the solutions become clear. Tailing is observed in all the chromatograms. It can be seen from the values recorded in Tables III and IV that the results are similar as in the case of nickel oxalate complexes. The R_F value reached a minimum with 1.2 equivalents of oxalate. In the case of cobalt oxalate complexes, when the concentration of $C_2O_4^{-2}$ is increased, most of the ions rise to the top and light tailing is observed from the starting point upto where the ion has moved to. The chromatograms were developed with H_2S water.

Ferric oxalate complex

In the case of ferric oxalate complexes there is no precipitation. Tailing is observed in chromatogram swith solutions containing up to 16 equivalents of the oxalate. With small additions of oxalate to the metal the R_F value does not change as in the case of copper and nickel complexes; with the addition of more oxalate ions the R_F value begins to increase until it becomes 0.87 with 3.2 equivalents of oxalate. In the case of dilute solutions the R_F value becomes 0.83 with 3.2 equivalents of oxalate. The chromatograms were developed with a dilute solution of potassium ferrocyanide containing 2% H_2SO_4. The indicator solution was always freshly prepared.

Ferrous oxalate complex

In these cases no oxalate is precipitated. No tailing is observed when the oxalate concentration is higher than nearly 7 equivalents. From Tables VII and VIII it can be seen that on the addition of oxalate to the metal the R_F value decreases and reaches a minimum with 2.4 equivalents of oxalate. On further increasing the proportion of oxalate the R_F value gradually increases and then has a tendency to become contstant. The chromatograms were developed with a freshly prepared solution of $K_3Fe(CN)_6$ containing 2% H_2SO_4.

SUMMARY

In this paper, oxalate complexes of Ni^{++}, Co^{++}, Fe^{++} and Fe^{+++} have been studied using the ascending filter paper strip method, with 50% ethanol as solvent. It was found in the case of Ni^{++}, Co^{++} and Fe^{++} that the R_F value decreases on the addition of increasing amounts of oxalate until it is nearly zero and then suddenly increases as complete redissolution of the precipitate occurs. Finally the values have a slight tendency to diminish again.

In the case of Fe^{+++} it was observed that the R_F value does not change on adding small amounts of oxalate. As the concentration of oxalate is increased, the R_F gradually begins to increase to a maximum and then has a slight tendency to diminish.

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CHROMATOGRAPHIC SEPARATION OF PYRUVIC, OXALACETIC AND ALPHA-KETOGLUTARIC ACID FROM TISSUE CULTURES

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INTRODUCTION

In investigating, with radioactive tracers, the operation of the Krebs cycle in normal and tumor tissues in culture, a specific determination of the individual α-ketoacids is essential.

Much work has already been published about the chromatographic separation of 2,4-dinitrophenylhydrazones (DNPH) derivatives of pyruvic, oxalacetic and α-ketoglutaric acid either on columns[1-3] or on paper[4-12]. In paper chromatography, the yellow DNPH derivatives show their positions without further development.

Starting from the methods used for sugars by CONSDEN, GORDON AND MARTIN[13] and by PARTRIDGE[14], paper chromatographic procedures have been elaborated. The methods have been derived from those of CAVALLINI, FRONTALI AND TOSCHI[4,5]. They are not satisfactory for the present purpose. MEISTER AND ABENDSCHEIN[7] have studied the paper chromatography of the 2,4-dinitrophenylhydrazones of 35 ketoacids separately, but not in mixture or after extraction from biological material. The R_F values given for the pyruvic, oxalacetic and α-ketoglutaric acid hydrazones make their separation from a mixture appear impracticable. Similarly the solvent system of SÝKORA AND PROCHÁZKA[8] makes possible the separation of ketoacids from other carbonyl compounds, but not their separation from each other. The methods introduced by EL-HAWARY AND THOMPSON[6], SELIGSON AND SHAPIRO[9], and MCARDLE[11] were worked out for the separation and identification of pyruvic, oxalacetic, α-ketoglutaric and acetoacetic acids in blood. However, in these methods carbonyl compounds other than ketoacids, possibly present in tissue, would interfere. Moreover, the high ammonia content (2.5 N) of the solvent system used by MCARDLE causes the decomposition of some material, presumable of other hydrazones; the decomposition products appear as brown lines, which interfere with the clear separation. In the method of KUN AND GRACIA-HERNANDEZ[10], the hydrazones of oxalacetic and α-ketoglutaric acid, as well as of other ketoacids, travel together in one slowly moving

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group. Therefore, these authors divided the paper chromatogram into several horizontal sections, extracted each strip singly, and subjected the different fractions to a second chromatographic separation. This complicated procedure results in a loss of material and is time consuming. Moreover, it was reported by some workers that the hydrazones of individual ketoacids are split up to give two or three or even four spots[7,10-12].

A search has therefore been made for a suitable method to separate pyruvic, oxalacetic, and α-ketoglutaric acids from tissue cultures. The method here described is derived from those of CAVALLINI[4,5] as well as of KUN AND GRACIA-HERNANDEZ[10]. It consists essentially of descending chromatography, on a filter paper freshly impregnated with 0.1 *M* phosphate buffer of pH 7.4, with a solvent consisting of *n*-butanol pre-treated with 3% aqueous ammonia.

EXPERIMENTAL

1. *Chromatography and estimation of the ketoacids*

The oxalacetic acid was prepared by the method of KRAMPITZ AND WERKMAN[15] from the sodium salt of diethyl oxalacetate. This had been made as described by WISLICENUS[16]. The α-ketoglutaric acid was prepared from ethyl oxalosuccinate according to WISLICENUS AND WALDMÜLLER[17]. Sodium pyruvate of Prolabo Laboratories was used. Oxalacetic and α-ketoglutaric DNPH were prepared by the method of HEIDELBERGER[18], pyruvic DNPH by the method of PRICE AND LEVINTOW[19].

The hydrazones were quantitatively separated from each other on Whatman No. 1 filter paper freshly impregnated with 0.1 *M* phosphate buffer of pH 7.4. For impregnation the paper sheet was immersed in a tray containing 400 ml buffer, and subsequently suspended vertically at room temperature for drying. Phosphate determination in the upper and lower halves of the impregnated paper showed that 1 cm² contained 138 and 140 μg phosphate, respectively. Buffer solution of higher concentration or of different pH value will not give a suitable paper. The DNPH derivatives, dissolved in 1 ml alcohol, were transferred quantitatively dropwise to the starting point at 15 cm from the upper end of the paper and dried in a current of hot air.

n-Butanol, previously equilibrated with an equal volume of 3% aqueous ammonia, was used as the solvent. More concentrated NH_3 decomposes some hydrazones to brown products so that no clear separation is obtained. The atmosphere of the glass tank was equilibrated with the solvent for at least 5 hours. Then the solvent was allowed to descend for 24 hours. The R_F values of the freshly prepared pyruvic, oxalacetic and α-ketoglutaric acid hydrazones were determined as 0.61, 0.10 and 0.07, respectively. However, if the pyruvic DNPH was kept—even in the refrigerator—for some time, a second spot, corresponding to an R_F value of 0.75 appeared. On application of the method to tissue cultures, the second pyruvic DNPH spot was always observed. For quantitative analysis both pyruvic DNPH spots were eluted together.

For elution, the spots were cut out and placed into 10 ml of 0.1 *M* phosphate buffer solution of pH 7.4, and left for 5 hours with occasional shaking. The yellow solutions were filtered, and their extinctions measured in a Lange colorimeter, model 5, with the filter BG 7 against a blank similarly prepared by cutting an equal area of empty paper from the chromatogram. The concentrations were derived from a calibration curve. The optical densities were found to follow the Beer-Lambert law.

2. *Measurement of radioactivity*

The radioactivity was measured by filling a gas Geiger counter with CO_2[20]. In a vacuum line, to which the counter was attached by a ground joint, the CO_2 was set free from the $BaCO_3$ with perchloric acid, dried with $Mg(ClO_4)_2$, and trapped in a U-tube cooled by liquid air. After all traces of air had been pumped off, the CO_2 was allowed to evaporate, and its pressure determined with a Hg manometer. Finally, the gas was frozen into the liquid air-cooled glass finger of the Geiger counter, the tap of the counter closed, and the CO_2 evaporated again.

The pressures in the counter were, as a rule, 200–400 mm Hg. Accordingly, the operating voltages varied between 3 and 5.5 kV. In most experiments plateaus of 200–400 V were easily obtained; in case of difficulty, 5% benzene vapour were added from a storage bulb. As no radiation is absorbed in a window or in the sample itself, and geometrical efficiency is essentially 4π, the counting yield is not far below 100%. To calculate the amount of radiocarbon in the sample from the count, corrections had to be applied for the (small) dead volume and for the incompleteness of the recovery. This was done by the formula:

$$A = (A_m - B)\, F\, G\, \frac{C}{100} \cdot \frac{760}{p} \cdot \frac{22.4}{v} \cdot \frac{T}{273} \,.$$

Here A is the total activity of the entire sample, A_m the measured activity, B the background activity, G the weight of the sample (in mg), C the percentage of carbon in the sample, p the pressure of the CO_2 (in mm Hg), v the volume (in ml) of the part of the apparatus connected with the manometer, and T the absolute temperature. The factor F (1/0.85 for the counting tube here used) allows for the dead volume of the counter.

In practice, the sensitivity of the method, with undiluted radioactive glucose, as obtained by radiophotosynthesis in usual conditions (about 100 μC/mg), is of the order of 10^{-8} mg. The sensitivity can still be improved by using glucose of higher specific activity, by counting for longer periods, or by reducing the background through enhanced shielding and an anti-coincidence circuit. On the other hand, the sensitivity is bound to be less when the radioglucose is diluted by inactive glucose, as in the experiments here described ($\sim 10^{-5}$ mg).

For the determination of the radioactivity of a substance contained in a spot of a chromatogram, the spot was cut out, placed into 10 ml water and left about 12 hours with occasional shaking. The yellow solutions were filtered. Determination of radioactivity in a spot could be carried out even when the amount of substance was too small to make the yellow colour visible, provided the position of the spots was known through authentic samples run in parallel.

In the cases of pyruvic and ketoglutaric DNPH the aqueous solutions were evaporated to dryness on the water bath with 37.3 mg inactive glucose as a carrier, and the residue combusted by the wet method of VAN SLYKE AND FOLCH[21]. To prevent decomposition of the oxalacetic DNPH, this was extracted from the yellow solution twice with 40 ml ether, this solution dried, in the presence of the inactive glucose carrier, under reduced pressure at room temperature, and the residue combusted. In all cases, the CO_2 obtained was absorbed by N NaOH, precipitated as $BaCO_3$, washed, dried and stored for measurement.

3. *Cultivation of tissue with radioglucose*

The chicken mesenchyma tissue ("fibroblasts") was taken from the legs of embryos 10 days old. The tissue was grown in roller tubes on a layer of chicken plasma covering the glass wall. The nutrient medium for the fibroblasts consisted of chick embryo extract, human ascites fluid and Gey's isotonic buffered salt solution in the ratio 4:40:56. The Gey solution contained 0.1% inactive glucose. The roller tubes were closed by rubber caps, but the supply of oxygen did not decrease appreciably during an experiment.

4–5 days after explantation, the fibroblasts were treated with trypsin at 37° and pH = 8.5 for 30–35 min as described previously[22]. Thereafter, the tissue was centrifuged and washed several times with salt solution. The cells were now suspended in chick embryo extract and counted in a haemocytometer. Finally, the required quantity of cells, with a volume of liquid not exceeding 0.1 ml, was inserted into roller tubes coated with plasma, and left 3–4 hours to settle. Then nutrient medium (1 ml) was added, and the tubes revolved at 14 r.p.h. in the drum. After a lag time, in which the cell count decreased to one quarter, the tissue began to grow and to form a monolayer of cells on the plasma surface. Within about 3 days, the original number of cells was reached again. At the end of an experiment, the tissue could be treated with trypsin, and the cells counted again.

In experiments with radioglucose, every tube was supplied with 1 ml of fresh medium and 0.4 μC of generally labelled glucose. The glucose had been prepared in Amersham from radioactive CO_2 by photosynthesis, and purified in our laboratories by ion exchange and paper chromatography[23]. The incubation with the radioactive solution was always carried out for 24 hours.

4. *Recovery of the ketoacids from the cultures*

In order to determine the percentage recovery from tissue cultures of each ketoacid alone or in mixture, known weights of the individual acids or known amounts of their mixture were added to the medium on top of the tissue, the acids extracted, separated by paper chromatography and estimated colorimetrically.

After addition of the ketoacid, the roller tube was cooled down immediately to 0°, the medium deproteinized by addition of 0.1 ml of ice-cold 60% $HClO_4$, and the contents transferred to a centrifuge tube. The roller tube was washed with alcohol and the wash alcohol added to the solution. After removal of the protein by centrifugation, the supernatant was filtered through Whatman No. 2 paper into a test tube containing 1 ml of a freshly prepared 0.1 M solution of 2,4-dinitrophenylhydrazine in 0.2 N alcoholic HCl. The mixture was kept at room temperature for two hours, thereafter transferred to a 100 ml separating funnel, and the mixture of the hydrazones extracted.

With ethyl acetate as extractant, an emulsion was obtained, and the complete separation into two layers met with difficulty. The use of a mixture of alcohol and ethyl acetate to prevent emulsification[11] was found tedious and wasteful. Therefore, ether was employed as the extractant. The test tube was washed three times with 15 ml ether, the wash ether combined with the bulk of the liquid, and about 150 ml water were added. After gentle shaking, a yellow ether layer containing all the hydrazones and a colourless aqueous layer separated. The aqueous layer was again extracted twice with ether (15 ml each time), the whole of the ether layer (about 60 ml) washed twice with 100 ml water, and the ether containing all DNPH derivatives re-extracted once with 2 ml 10% Na_2CO_3 in order to separate the DNPH derivatives of ketoacids from those of other carbonyl compounds. A repetition of extraction with carbonate is unnecessary, and only increases the alkaline decomposition of DNPH. The alkaline extract was acidified with dilute HCl and twice re-extracted with ether. The whole ether extract (10 ml) was washed twice with 20 ml water and dried by filtration through anhydrous Na_2SO_4. Finally the ether was evaporated, the hydrazones redissolved in 1 ml alcohol, transferred quantitatively to the starting point and separated chromatographically.

RESULTS

1. *Recovery of ketoacids added to cultures*

To determine the recovery of the individual ketoacids from nutrient medium, 0.1 ml of a freshly prepared 0.04% solution of one ketoacid was stirred into the medium of each tube, and the medium immediately worked up as described (Table Ia). In another set of experiments, 0.3 ml of a mixture of equal parts of 0.04% solutions of all three ketoacids were added to each tube, and the medium again worked up as described (Table Ib). In every case, the "natural" ketoacid content of the medium (blank value), as determined in parallel experiments, was deducted.

While figures for ketoglutaric DNPH appear satisfactory, about one half of the oxalacetic DNPH is lost. The "gain" in pyruvic DNPH (Table Ib) leads to the suspicion that oxalacetic DNPH may be decarboxylated during the procedure and thus may yield additional pyruvic DNPH. Therefore, the stability of oxalacetic DNPH in the given experimental conditions was investigated. In particular, the influences of (a) heat, and (b) alkali were studied.

(a) The ether solution of the extracted acidic phenylhydrazones had been evaporated by hot air, and the substances had again been exposed to hot air wheer the hydrazones were applied to the starting line on the paper. To test the influence of the heat, 0.1 ml of a freshly prepared 0.04% alcoholic solution of pure oxalacetic

DNPH solution was subjected to this procedure. On chromatography, in addition to the spot of oxalacetic DNPH, the spots of pyruvic DNPH appeared. By colorimetry, it was found that 49% of the oxalacetic acid had decomposed to pyruvic acid.

(b) The acidic phenylhydrazones had been separated from other phenylhydrazones with 10% Na_2CO_3 solution. To test the influence of alkali, 0.1 ml of a freshly prepared 0.04% alcoholic solution of oxalacetic DNPH was dissolved in 2 ml 10% Na_2CO_3 solution. The mixture was left for 10 minutes, acidified with 4 ml 50% HCl, and twice extracted with ether. The ether extract (15 ml) was washed with water, dried by filtration through anhydrous Na_2SO_4, and the ether evaporated under reduced pressure without applying heat. The resulting residue was dissolved in 0.5 ml alcohol, transferred to the starting line, and dried with a current of cold air. On chromatography, no pyruvic DNPH spot was observed.

TABLE I

RECOVERY OF THE KETOACIDS

Acid	*Number of expts.*	*Acid added (μg)*	*Acid recovered (μg)*	*Blank values (μg)*	*Net recovery (μg)*	*Net recovery (%)*
			(a) Recovery of the individual ketoacids			
Pyruvic	15	40	76	41	35	88
Oxalacetic	20	40	17	0	17	43
α-Ketoglutaric	11	40	37	2	35	88
			(b) Recovery of the mixed ketoacids			
Pyruvic	15	40	93	41	52	130
Oxalacetic	15	40	17	0	17	43
α-Ketoglutaric	15	40	37	2	35	88

The last result indicates not only stability of oxalacetic DNPH in alkali, but also lack of decomposition during treatment and chromatography at room temperature. On the other hand, major errors must have occurred in previous investigations where oxalacetic DNPH had been exposed to heat during analysis. Consequently, in the following experiments, the effect of heat was avoided by producing the hydrazone in the cold and by using air of room temperature only during the application of the alcohol solution to the filter paper.

2. *Location of the ketoacids*

Obviously, the oxidation of substrates by way of the citric acid cycle proceeds within the tissue. Consequently, to gain insight into the mechanism of respiration by tissue in culture, it is essential to know whether the ketoacids remain within the tissue or whether they diffuse to the nutrient medium. Therefore, the ketoacids were determined in (a) nutrient medium alone after incubation, (b) supernatant medium after incubation with tissue, and (c) tissue alone after incubation with medium. This was done in parallel as follows.

(a) After incubation, 5 ml of the nutrient medium were deproteinized at 0° by the addition of 1 ml ice-cold 60% perchloric acid and centrifuged 30 min at 7000 r.p.m. and at 0°. The clear supernatant was kept with dinitrophenylhydrazine solution (5 ml) for 3 hours at 0°, the ketoacids were extracted, separated by paper chromatography and determined colorimetrically.

(b) 5 tubes containing 1 ml nutrient medium each were cooled immediately after incubation to 0°, the nutrient medium from each culture decanted separately, the tissue quickly rinsed twice with 1 ml saline solution, and the saline washings combined with the medium. Deproteinization and analysis were carried out as described above.

(c) The culture tissue in each tube of experiment b was frozen in dry ice and homogenized, after adding 1 ml water, at 0° with quartz sand for 5 min by using a rapidly rotating glass pestle, the diameter of which was only slightly inferior to that of the tube. The homogenate was deproteinized by adding 0.1 ml ice-cold perchloric acid and centrifuged at 0° for 30 min at 7000 r.p.m. The clear supernatant of each culture was kept at 0° with 1 ml dinitrophenylhydrazine solution for 3 hours, the ketoacids extracted, chromatographed and determined.

The mean values obtained are given in Table II. The three vertical colums refer to the experiments outlined under a, b and c, respectively.

TABLE II

LOCATION OF THE KETOACIDS

Acid	*Medium incubated alone (μg/ml)*	*Tissue incubated with medium (per tube)*	
		Within medium (μg)	*Within tissue (μg)*
Pyruvic	16	39	7
Oxalacetic	0	0	0
α-Ketoglutaric	2.2	2	0

By deducting the mean pyruvic acid content of the nutrient medium, incubated by itself, from the mean content in the incubated medium decanted from the culture, it is found that on balance 23 μg pyruvic acid had diffused from the tissue to the culture medium. Neither ketoglutaric nor oxalacetic acids were present within the tissue in detectable amounts.

In a further set of experiments, the distribution of the radioactive ketoacids, after incubation of $\sim 5 \cdot 10^5$ cells $\sim$ 0.3 mg dry tissue with radioglucose, was determined. Compared with the mass of the inactive glucose in the tube ($\sim$ 1 mg), the mass of the 0.4 μC of radioactive glucose ($\sim$ 1.2 μg) was negligible.

The possibility of a production of radioactive ketoacids from radioglucose by nutrient medium alone had to be considered. Therefore, blank experiments were carried out by incubating medium, without tissue, in parallel with medium plus tissue. However, the radioactivity of the pyruvic acid (28 d.p.m.) was negligible, and that of oxalacetic and ketoglutaric acid was even less.

Much activity, however, was found in the ketoacids after incubation of tissue with medium. All figures in Table III are mean values from three runs. The quantities of the ketoacids have been calculated from their activities by assigning their carbon atoms specific activities identical with that of the carbon atoms in the total glucose initially present (approximately $2.2 \cdot 10^6$/mg C). It is seen that the larger part of the radioactive pyruvic acid, but not of the oxalacetic or ketoglutaric acid, had diffused from the tissue to the supernatant medium. The quantity of the pyruvic acid according to Table II is far higher than according to Table III; in the case of ketoglutaric acid, the distribution according to Table III is the opposite of that according to Table II. These differences may be attributed to the non-radioactive acids present before the addition of radioglucose and/or derived from other precursors than glucose.

TABLE III

LOCATION OF THE RADIOACTIVE KETOACIDS

Acid	*Within medium*		*Within tissue*	
	Activity (d.p.m.)	*Quantity (μg)*	*Activity (d.p.m.)*	*Quantity (μg)*
Pyruvic	9200	3.4	595	0.22
Oxalacetic	0	0	640	0.27
α-Ketoglutaric	0	0	1075	0.39

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SUMMARY

A new method for the isolation and separation of α-ketoacids participating in the citric acid cycle is described. The dinitrophenylhydrazones (DNPH) formed in the cold aqueous system are extracted with ether, removed from the ether with sodium carbonate, re-extracted with ether after acidification, and subjected to descending chromatography on a paper impregnated with phosphate buffer. The solvent consists of *n*-butanol pre-treated with an equal volume 3% aqueous ammonia.

Pyruvic DNPH gives two spots ($R_F = 0.61$ and 0.75), oxalacetic and α-ketoglutaric DNPH each one spot only ($R_F = 0.10$ and 0.07, respectively). After elution, the hydrazones are determined by colorimetry. Two micrograms of the acids can still be determined, and a check on recovery is possible by isotope dilution.

Oxalacetic DNPH is stable at room temperature, even when dissolved in 10% Na_2CO_3, but is decomposed to pyruvic DNPH by moderate heat. Therefore, in previous investigations where oxalacetic DNPH had been exposed to heat during analysis, major errors must have occurred.

After incubation, for 24 hours, of about 0.3 mg (dry weight) of chicken mesenchyma ("fibroblasts") in tissue culture most of the pyruvic and the ketoglutaric acids were found in the medium, while oxalacetic acid was below the limit of detection. After incubation with radioglucose, most of the radioactive pyruvic acid was found to have diffused into the medium but radioactive ketoglutaric and oxalacetic acids were detected within the tissue only.

Note added in proof.

A further study of the ketoacids in blood has now been published, and three fast moving spots identified[24]. Three fast moving spots have also been observed on our chromatograms, but not identified.

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SHORT COMMUNICATIONS

The quantitative colorimetric determination of colchicine in aqueous solution, and studies on its application to urine*

The oral administration of colchicine to relieve acute attacks of gout has been practized for many years. Few studies of its metabolism and excretion have been made in human subjects. After intravenous injections of relatively large doses to rats, BRUES[1] has reported the presence of colchicine in their urine, as determined by the chemical technique of BOYLAND AND MAWSON[2]. This technique, however, is not sufficiently sensitive to detect smaller excretion which would be expected in human subjects after a therapeutic dose of from 4 to 6 mg *per diem*. In the present study, improved methods for the extraction, separation and colorimetric estimation of colchicine have been developed. The procedure has yielded good recoveries of colchicine added to water or to extracts of urine. Colchicine added directly to urine was incompletely recovered, suggesting that an alteration in its properties had occurred.

METHODS AND RESULTS

Materials

1. Colchicine** purified according to the procedure of ASHLEY AND HARRIS[3]. Recrystallization from ethyl acetate yielded pale yellow crystals, m.p. 151–152° (uncor.).
2. Colchiceine prepared by the method of SORKIN[4] from colchicine yielding a white crystalline material, m.p. 168–170° (uncor.).
3. Benzene, redistilled.
4. Ethylene dichloride, redistilled.
5. Formamide, C. P., stored in desiccator over concentrated H_2SO_4.
6. Whatman No. 1 and No. 4 paper for chromatography were previously washed for seven days in ethanol and seven days in water by descending chromatography.

Procedures

Colorimetric determination of colchicine. In the existing procedures (BOYLAND AND MAWSON[2]; KING[5]), colchicine is hydrolyzed to yield colchiceine, which can readily be determined by means of the olive green color it gives upon addition of ferric chloride. The absorption spectrum of this complex exhibits maxima at 470 and 630 mμ. In order to minimize the absorption of brown-colored extraneous material in urine

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* This research was supported by funds granted by the Lillia Babbitt Hyde Foundation, New York, N.Y.

** Obtained from S. B. Penick & Co., 50 Church Street, New York 7, N.Y.

extracts, we have measured optical densities at 630 rather than at 470 mμ. Conditions of hydrolysis which give almost 100% conversion to colchiceine have been adopted.

Various quantities of colchicine (from 100 to 1000 μg) were dissolved in 1 ml of N HCl in 50% ethanol and transferred to 15 mm by 150 mm test tubes. The test tubes were covered with tare bulbs, and placed in a boiling water bath for 30 min. To each tube was then added 0.5 ml of distilled H_2O, 2 ml of $N/10$ aqueous HCl, and 0.01 ml of 10 M $FeCl_3$. The resultant solutions are read at 630 mμ in a Coleman Model 6B Spectrophotometer. Table I shows that the resulting optical densities

TABLE I

THE ESTIMATION OF COLCHICINE BY HYDROLYSIS AND REACTION WITH FERRIC CHLORIDE

µg	*Optical density**		*% conversion****
	colchicine	*colchiceine***	
100	0.121	0.123	98.4
200	0.243	0.245	99.2
300	0.360	0.369	97.6
400	0.485	0.490	99.0
500	0.601	—	—
1000	1.23	—	—
		Average:	98.6

* Average of three results each.

** Colchicine is converted to colchiceine with a 92% theoretical yield. The optical density of colchiceine was corrected by using 92% of the density obtained.

*** % conversion is equal to the per cent of the theoretical yield (92%) obtained.

TABLE II

EXTRACTION OF COLCHICINE FROM AQUEOUS SOLUTIONS AT VARIOUS pH'S WITH DIFFERENT SOLVENTS

Solvent	*pH*	*Average % recovered**
Chloroform	7	60
	4	79
	9	72
Benzene	5	35
	9	34
	7	34
Ethyl acetate	5	62
	9	59
	7	62
Ethylene dichloride	5	87
	9	85
	7	94
	7**	88
	7***	81

* Based on three experiments.

** 20 ml portions of solvent.

*** 10 ml portions of solvent.

followed Beer's Law, and that an average 98.6% of the theoretical conversion to colchiceine was obtained.

Extraction of colchicine from water. 40 ml aqueous solutions, containing 1 mg of colchicine, adjusted to specified pH values with HCl or NaOH were extracted three times with 30 ml portions of solvent. The residues from the combined extracts, after evaporation, were assayed for colchicine. Table II shows that extracting with ethylene dichloride at pH 7.0 was the most efficient. A decrease in the ratio of solvent to aqueous medium volume showed a decrease in efficiency of extraction.

Since 1 *N* NaOH removes much of the colored material from the ethylene dichloride extracts of urine, the loss of colchicine by such washes was studied.

A 40 ml aqueous solution containing 1 mg colchicine at pH 7 was extracted three times with 30 ml volumes of ethylene dichloride. The combined extracts were washed twice with 10 ml of *N* NaOH and once with 10 ml ethylene dichloride. The total combined ethylene dichloride extracts were assayed for colchicine. It may be seen (Table III) that a loss of about 4% occurred as a result of the NaOH washes.

TABLE III

EFFECT OF AQUEOUS SODIUM HYDROXIDE WASH ON ETHYLENE CHLORIDE EXTRACTS OF COLCHICINE SOLUTIONS

Sample	*% colchicine recovered*
1	88.8
2	89.7
3	90.5
Average:	89.7

TABLE IV

RECOVERY OF COLCHICINE IN THE EFFLUENTS OF 36 cm LONG CHROMATOGRAMS DEVELOPED BY THE ZAFFARONI TECHNIQUE[6] USING A BENZENE–FORMAMIDE SYSTEM

The effluents were evaporated and determined for colchicine.

Chromatogram No.	*Whatman paper No.*	*Days collected**	*Colchicine chromatographed µg*	*Colchicine recovered µg*	*Colchicine recovered %*
1	1	2–12	600	546	91
2	1	2–12	600	603	101
3	1	2–12	600	611	102
4	1	2–12	400	367	92
5	1	2–12	400	384	96
6	1	2–12	400	373	93
7	4	1– 7	1000	968	97
8	4	1– 7	1000	984	98
9	4	1– 7	1000	952	95
10	4	1– 7	1000	936	94
				Average recovery:	96 ± 3%

* Days of chromatographic development during which effluent was collected.

When colchicine was added to such washed extracts of urine, only 50–70% recovery was obtained after hydrolysis and colorimetry.

Paper chromatographic separation

In the belief that recoveries of colchicine from urine extracts could be improved by separation of the remaining pigments and other neutral materials, chromatographic fractionation of colchicine was undertaken. It was found that colchicine could be effectively chromatographed in the benzene–formamide system developed by Burton, Zaffaroni and Keutmann[6], on either Whatman No. 1 or No. 4 paper (Table IV). On the No. 1 paper, colchicine has a mobility of 0.4 cm per hour, which enabled it to be adequately separated from polar urine material left at the origin and a low-polarity pigment fraction that moved with the solvent front. The presence of colchicine on the paper could be shown by testing with concentrated HCl, whereupon a yellow-green band was obtained. However, the colchicine could not be quantitatively eluted from the paper with a variety of solvents (*i.e.*, ethanol, methanol, chloroform and ethyl acetate) and techniques (*i.e.*, macerating and pulverizing the papers in the hot solvents), all of which gave low recoveries. Recourse was therefore had to longer periods of chromatography, combined with a shorter strip of the faster No. 4 paper, in hopes that the colchicine would be found in the effluent. In the final procedure, the first effluents, containing the fast moving pigments, were discarded. Collections were then made after various periods of chromatography. Table V shows that good recoveries of colchicine added to urine extracts were obtained.

Application of techniques to urine specimens. On the basis of the above experiments, a method for the extraction of colchicine from urine was proposed. A 1 l specimen of normal urine containing colchicine, adjusted to pH 7, was extracted three times with 750 ml of ethylene dichloride. The combined extracts were washed twice with 225 ml

TABLE V

RECOVERY OF 1 mg OF COLCHICINE AFTER ADDITION TO EXTRACTS OF 1000 ml OF NORMAL URINE

Effluents from the chromatogram in a benzene–formamide system were collected after the low polarity urine pigments had visibly descended from the paper*, and were determined for colchicine.

1000 ml normal urine extract from urine pool No.	*Whatman paper No.*	*Length of paper (cm)*	*No. of days effluent collected*	*Average % colchicine recovered***
1	1	36	10	80
2	1	36	10	77
3	1	36	10	80
1	1	36	20	100
2	1	36	20	99
3	1	36	20	91
4	1	16	10	87
5	1	10	10	87
6	4	5	7	87

* A 36 cm Whatman No. 1 paper about 1 h, and a Whatman No. 4 paper about 20 min.

** Based upon two experiments.

of *N* NaOH and once with the same volume of water. The combined washes were backwashed with 500 ml of ethylene dichloride. This was combined with the previous ethylene dichloride extract and distilled on a steam bath *in vacuo*. The residue was chromatographed as previously described, on a 5 cm Whatman No. 4 paper. The seven day effluent was assayed for colchicine by evaporation, hydrolysis and reaction with ferric chloride.

When 1 mg of colchicine was added to two separate urine specimens and processed in the above manner, recoveries of only 6 and 9% were obtained. Since colchicine had been efficiently extracted from water, and recovered from extracts of urine previously prepared by the identical procedure, this failure was not readily explained. More vigorous extraction procedures were tried, in hopes of overcoming an apparent lower extractability of colchicine from urine than from water. However, neither *n*-butanol nor continuous ether extractions altered the final recoveries substantially. It must be concluded that an unknown alteration in the properties of colchicine results when it is added to urine.

DISCUSSION

The data presented show that the analytical techniques employed to extract colchicine from urine are valid. The poor, final results obtained must be attributed to some modification of the partition relations of colchicine. This hypothesis would explain the contradictory results obtained by BRUES[1] and LETTRÉ[7].

BRUES, using rat urines, estimated colchicine by the method of BOYLAND AND MAWSON[2]. This entailed hydrolysis of the urine in *N*/10 HCl at 100° for one hour and estimation of the colchicine as colchiceine by extraction with chloroform and reaction with ferric chloride. LETTRÉ, using extracts of unhydrolyzed urines, found an absence of the mitotic inhibiting effect on chicken heart fibroblasts associated with colchicine. The difference between their results may possibly be related to the additional hydrolysis procedure. It is well known that various conjugates are decomposed by acid hydrolysis. Analogously, this could explain BRUES' recovery and LETTRÉ's lack of recovery of colchicine from urine.

In the present study hydrolysis before extraction was not performed, since the resulting extract could not be washed with NaOH without removing the phenolic-like colchiceine, as well as extraneous matter and pigments. Further studies are necessary before the phenomenon observed can be understood.

The author is grateful to EDWARD F. HARTUNG, M.D., HILDEGARD WILSON, Ph.D., and MARGARET NEIL, for encouragement, advice and technical suggestions.

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Ein neues papierchromatographisches Verfahren

Sowohl absteigende, aufsteigende, als auch horizontale Trennungen finden ihre Grenzleistung in der Steighöhe, bzw. Laufstreckenlänge.

Auf Grund einer einfachen Überlegung und der sich aus dieser ergebenden Technik ist es möglich, den Wanderweg eines Gemisches beliebig zu verlängern und dadurch die Trennung seiner Komponenten im Vergleich zu den bisher möglichen Ergebnissen um 100% zu verbessern.

Die Versuche wurden aus gegebenem Anlass mit Aminosäuregemischen und Fruchtsäuregemischen vorgenommen. Die Trennungen erfolgten nach der horizontalen Methode unter Benutzung eines Rundfilterentwicklungsgerätes.

Technik

Der Durchmesser des Rundgerätes betrug 30 cm, das Papier wurde in Rechteckform von 30 cm Breite und 45–60 cm Länge geschnitten. Das Laufmittel—in unserem Fall *n*-Butanol–Ameisensäure–Wasser—kam in ein Schälchen innerhalb des Gerätes.

Den Laufmitteltransport besorgte ein Röllchen (Docht) aus dem gleichen wie zum Chromatogramm verwendeten Papier. Es wurde aus etwa 10 bis zu 30 cm langen und je nach gewünschter Länge des Tauchdochtes 4 bis 5 cm breiten Papierstreifen gerollt. Die Dochtstärke betrug bis zu 1 cm. Das zu untersuchende Gemisch hatte seinen Startpunkt in der mittleren Längsachse des Chromatogramms, etwa 2–3 cm vom Saugdocht entfernt. Dieser sitzt beim Anlaufen in der Mitte des Gefässes, ist also bei dem gegebenen Durchmesser des Entwicklungsgerätes dessen Radius entsprechend 15 cm vom Schmalrand des Papierrechtecks entfernt und genau in dessen Mittelachse. Er taucht dort in das mit dem Laufmittel gefüllte Schälchen. Sobald die Laufmittelfront den kreisförmigen Gefässrand erreicht hat, wird das Schälchen nachgefüllt und aus der Gerätmitte an dessen Peripherie in "Randdochtstellung" geschoben. Der Papierbogen wird in Richtung seiner Längsachse mitverschoben und mit dem gleichen Docht nunmehr aus Randdochtstellung weitergetrennt. Durch diesen Vorgang ragt ein Teil des feuchten Papierbogens aus dem Gerät heraus und wird abgeschnitten. Gleichzeitig ist ein entsprechendes Stück des noch nicht durchlaufenen, trockenen Papierrechtecks auf der gegenüberliegenden Geräteseite in dessen Inneres gezogen worden. Es ist darauf zu achten, dass Gerät und Deckel breite, horizontale und plangeschliffene Ränder haben. Nach Durchlaufen der jetzt im Gerät befindlichen Papierfläche kann, diesmal allerdings erst nach Trocknung, ein neuer Docht in vorgeschobener Stellung eingesetzt und aus Randdochtposition weitergetrennt werden. Der Start erfolgt also mit Mitteldocht, den Weiterlauf besorgen Randdochte, welche entsprechend den jeweils erreichten "Zwischenstationen" etwa 2–3 cm von diesen entfernt neu einzusetzen sind. Auf diese Weise entfernen sich die Komponenten immer weiter voneinander und ist eine Grenze nur durch die eventuelle Verdünnung gesetzt. Es kann auch mit schmaleren Papierstreifen und sofort aus Randdochtstellung gearbeitet werden. Dies richtet sich nach Substanzmenge, gewünschter Profilierung und mitlaufenden Tests. Aus Mitteldochten ergeben sich bogenförmige, verdünnte, aus Randdochten und schmaleren Papierstreifen massivere, fleckenförmige Transporte. Es sind Variationsmöglichkeiten gegeben, diese Mitteilung will nur Grundsätzliches weitergeben.

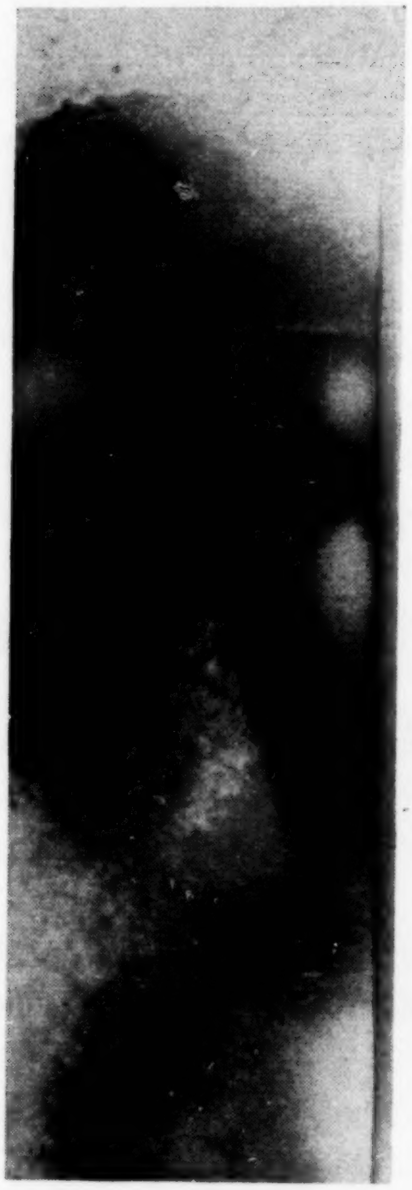

Fig. 1. Neue Methode.

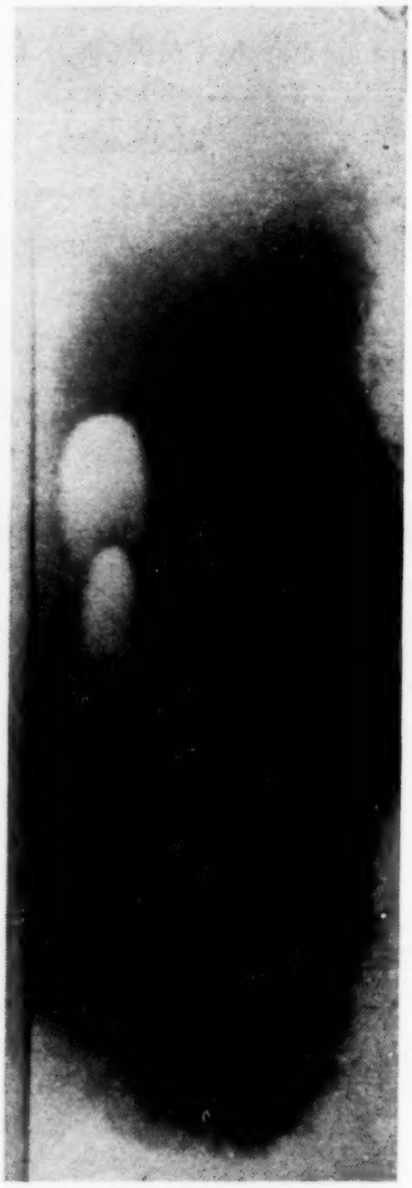

Fig. 2. Alte Methode.

Dem Verfasser gelangen scharf trennende Zwischenzonen von 8–10 mm, wo vorher unter gleichen Bedingugen, jedoch ohne Wanderdocht, im üblichen Ringchromatogramm bestenfalls 3–4 mm breite Trennzonen entstanden.

Diese neue Methode ist vor allem bei quantitativen Aufgaben, die mit Elution der getrennten Komponenten zu arbeiten haben, ein Fortschritt.

Bundesanstalt für Qualitätsforschung pflanzlicher Erzeugnisse, Geisenheim/Rhg. (Deutschland)

H. HALBENSTEINER

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Appearance of artifacts on chromatograms of quaternary ammonium compounds

In the course of extracting quaternary ammonium compounds from the electric organ of *Electrophorus electricus* (L.), aqueous extracts of the tissue were deproteinized with trichloroacetic acid and then extracted with ether. When the purified solutions were subsequently applied to the paper and the chromatograms run in alkaline solvents, it was found that the residual trichloroacetic acid caused the appearance of an artifact in the chromatographic pattern of the quaternary ammonium compounds present.

This alteration was observed in the case of choline and of several synthetic curares used in the study of the phenomenon of curarization in the eel. They were: 606 H.C.* (hexamethylene-bis(carbamyl choline) diiodide), 336 H.C.* (1,4-bis-(2-piperidinoethyl)-piperazine diethiodide), and F 2559 (Gallamine triethiodide, also known as Flaxedil).

Solutions of these compounds were prepared containing increasing amounts of trichloroacetic acid, then chromatograms were run in order to determine the amount necessary for appearance of the artifact.

The following alkaline solvent mixtures were used: ethanol–ammonia, propanol–ammonia–water, and butanol–pyridine–water.

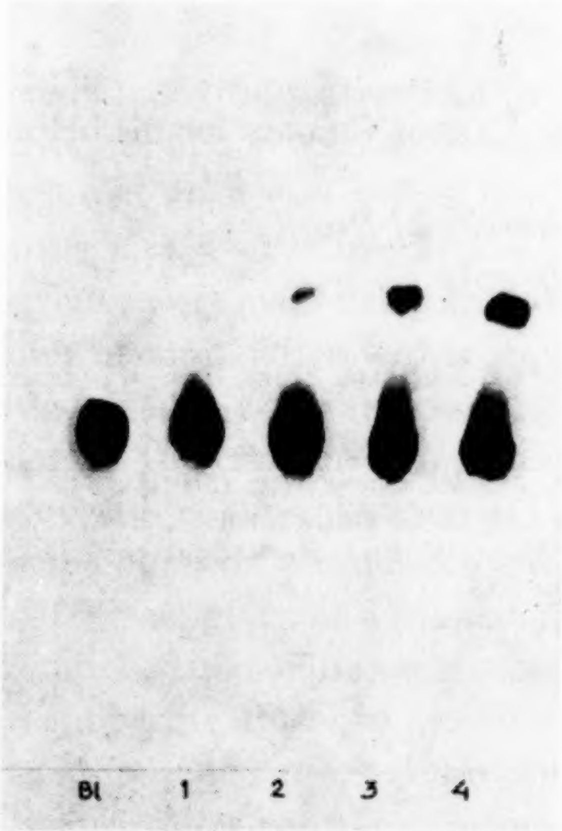

Fig. 1. Descending chromatogram run in ethanol–ammonia (95:5)[4]. Spot B1 contains 1 μmole of choline chloride alone. The other spots contain the same amount of choline chloride to which increasing amounts of trichloroacetic acid had been added: spot 1 contains 0.2%, spot 2 0.3%, spot 3 0.4% and spot 4 0.5% trichloroacetic acid. R_F choline = 0.54; R_F artifact = 0.79.

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* Kindly supplied to Prof. CARLOS CHAGAS by Prof. JEAN CHEYMOL.

Reagents which are commonly employed to develop choline and curares on paper also reacted with the artifact. We used iodine vapours as well as reagents containing bismuth subnitrate[1], bismuth carbonate[2], chloroplatinic acid[2] and phosphomolybdic acid[3].

The size of the artifact spot increased at the expense of the principal spot.

The synthetic curares 606 H.C. and 336 H.C. showed an additional spot in all three solvents when 0.2 to 0.4% trichloroacetic acid was present. At these concentrations of trichloroacetic acid, the artifact appeared in chromatograms of F 2559 run in butanol–pyridine-water and in propanol–ammonia, but not when ethanol–ammonia was the solvent, whereas in the case of choline chloride solutions, containing the same increasing amounts of trichloroacetic acid, the artifact appeared only when ethanol–ammonia was used.

When the artifact was eluted and rechromatographed in the same solvent, or in acid solvents (such as butanol–acetic acid or butanol–hydrochloric acid) it reappeared as such, and not as the parent quaternary ammonium compound.

Other authors[5,6,7] have reported trichloroacetic acid-produced artifacts in chromatograms of adrenaline, histamine and thyroxine when acid solvents were used, while we observed the phenomenon in the case of quaternary ammonium compounds when the solvents were alkaline.

This effect of trichloroacetic acid occurs frequently enough to suggest that care should be taken in the preparation and interpretation of chromatograms of substances extracted by procedures involving use of this agent, even when only very small amounts of it remain.

Thanks are due to Professor CARLOS CHAGAS for his helpful support.

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Extraction of ^{35}sulphur from pile-irradiated potassium chloride

Introduction

^{35}S (half-life 88 days) is widely used as a tracer in agricultural and biochemical studies. It is generally obtained in the carrier-free state by the ^{35}Cl $(n, p)^{35}$S reaction using KCl as the target material. ^{35}S thus produced is radiochemically contaminated with ^{32}P (formed by the ^{35}Cl$(n, \alpha)^{32}$P reaction), from which it must be separated.

A number of methods have been reported by different authors[1,2,3] for the separation of ^{35}S from various targets, *viz.* CCl_4, KCl, NaCl, $FeCl_3$ etc. In the conventional method[4], followed in many countries, the pile-irradiated KCl is dissolved in water and the solution passed down a cation-exchange column; K^+ ions are adsorbed on the column while ^{32}P and ^{35}S pass out with the effluent solution as phosphate and sulphate ions. ^{32}P is then removed either by coprecipitation with $La(OH)_3$, or by passage through a column containing aluminium shavings.

This paper describes a new method for the separation of ^{35}S from pile-irradiated KCl in which the separation of carrier-free ^{35}S has been much simplified through the use of an anion-exchange column. The separation of ^{35}S from ^{32}P and K^+ is effected in a single step and ^{35}S is obtained in a radiochemically pure form. The method has been tried with success for the isolation of millicurie amounts of ^{35}S and is being adopted for the routine production of this isotope at the Trombay Establishment.

Experimental details

15 g samples of KCl were irradiated with neutrons, in the Apsara reactor, Trombay, at a flux of $3 \cdot 10^{11}$ n/cm^2/sec for about 100 h. The ^{42}K (half-life 12.4 h) formed by the ^{41}K$(n, \gamma)^{42}$K reaction was allowed to decay before processing. A negligibly small amount of ^{36}Cl is formed during such a short irradiation.

Measurements of radioactivity were made using an end-window type G.M. counter (window thickness 2.5 mg/cm^2) in conjunction with a scaling unit.

The irradiated KCl was dissolved in water and made up to a known volume, which served as a stock solution for all the experiments. A suitable aliquot was pipetted out and diluted with an appropriate volume of water, so as to give a solution approximately 0.1 M in KCl. A small amount of phosphate carrier (5 mg) is added in order to reduce (i) the adsorption of ^{32}P on glass, and subsequently (ii) the contamination of ^{35}S by ^{32}P during the acid elution. This solution was passed down an anion-exchange column containing the resin Amberlite IRA-400 (40–60 mesh) in the chloride form. The effluent was collected separately and checked for any activity. The column was next washed with 100 ml distilled water and the effluent again checked for activity. A certain amount of ^{32}P was detected in both these fractions, indicating that it is poorly fixed on the column. Finally the column was eluted with 0.1 M HCl and fractions of about 50 ml each were collected separately. The activity in each of these fractions was measured. 0.2 ml from each fraction was deposited on a glass planchet and then evaporated to dryness under an infra-red lamp. They were counted in an end-window G.M. counting assembly, first without an absorber, then with an aluminium absorber of thickness 30 mg/cm² interposed between the source and the counter window. Such an absorber cuts off all β radiations from ^{35}S (E_{max} 0.17 MeV), so that any contamination due to ^{32}P (E_{max} 1.7 MeV) would easily be detected. It was found that the first two or three fractions (100–150 ml) contained the ^{32}P free of any ^{35}S, and that the later fractions contained pure ^{35}S. A typical elution curve is shown in Fig. 1. The curve shows two distinct peaks with good resolution, which indicates the possibility of separating radiochemically pure, carrier-free ^{35}S from irradiated KCl using an anion exchanger.

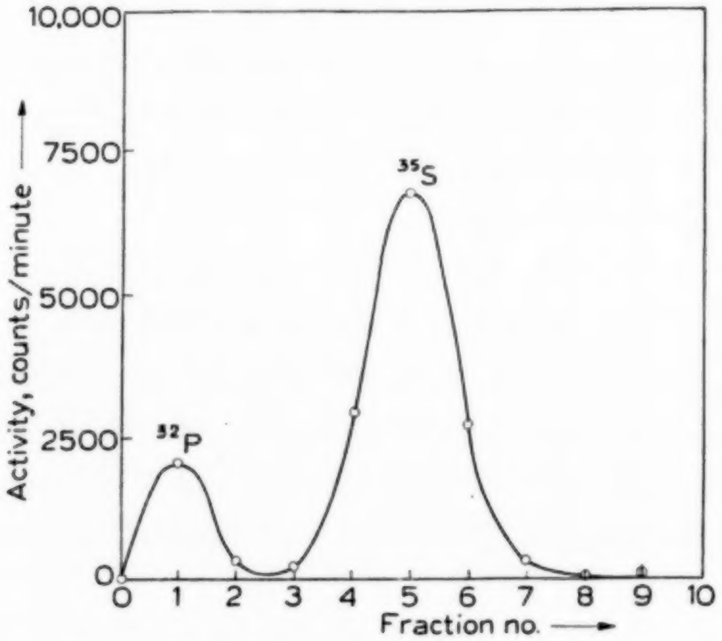

Fig. 1. Elution curve with 0.1 *N* HCl.

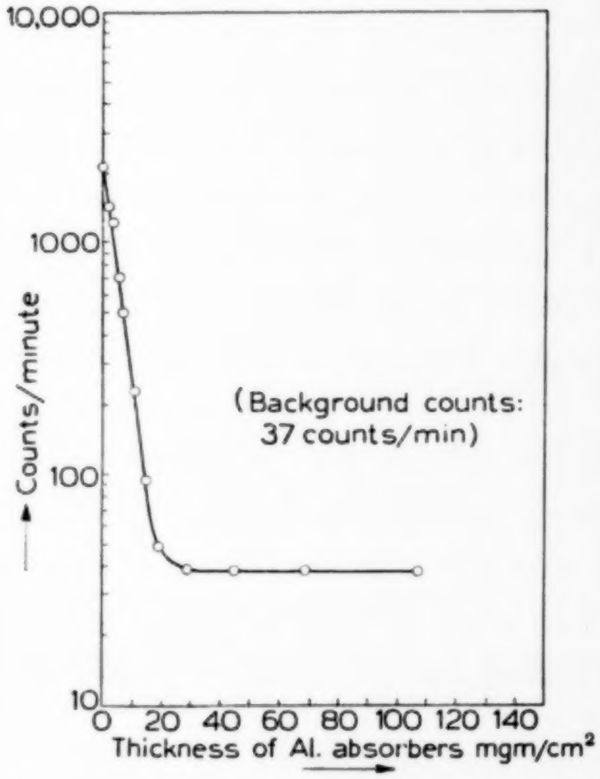

Fig. 2. Aluminium absorption curve of the final ^{35}S solution.

The recovery of ^{35}S has been found to be almost quantitative. The column was checked for any residual activity on it by elution with concentrated HCl. No activity was detected in the effluent solution.

Radiochemical purity

In Fig. 2 is shown the aluminium absorption curve taken on an aliquot from a mixture of all the fractions containing pure ^{35}S. It is evident that the ^{35}S obtained is radiochemically pure.

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BOOK REVIEW

Trace Analysis, Papers presented at a Symposium on Trace Analysis held at the New York Academy of Medicine, New York, November 2nd–4th, 1955, edited by J. H. YOE AND H. J. KOCH JR. John Wiley & Sons, Inc., New York, 1957, 672 pp., price $ 12.

The symposium is divided into three parts: "Methodology", "Instrumentation" and "Sensitivity, Separation, Concentration and Contamination". The first three chapters of "Methodology" deal with chromatographic methods. Chapter 1 by H. H. STRAIN entitled Chromatography and Electrochromatography (27 pp.) attempts to define, classify and summarise the numerous chromatographic and electrophoretic methods. This is a difficult and thankless task unless sufficient detail and explanation is devoted to each method. Unfortunately Dr. STRAIN has tried to present the different methods in a series of tables, which are of no help to the beginner. Also it appears to be impossible to include all techniques in such tables. For example Table VIII lists the electrochromatographic methods and the reader must conclude that electrophoresis in troughs is never carried out with gels as a supporting medium or that electrophoresis in tubes is usually carried out with gels and not with starch or cellulose although the contrary is true for both cases. The main shortcomings of this review thus seem to be that too much is compressed into too little space.

Chapter 2 by K. A. KRAUS on Ion Exchange is easily the clearest and best introduction to ion exchange chromatography known to the reviewer. Especially the theory of ion exchange has been presented with remarkable skill. This chapter may be recommended as text for students as well as for research workers intending to use ion exchange techniques.

The third chapter entitled "Extraction" by L. C. CRAIG deals essentially with the theory of counter current distribution. It is extremely well written and contains a lot of stimulating material for the partition chromatographer such as the shape of distribution patterns with non-linear distribution isotherms. The fractionation of bacitracin fragments by counter current distribution is given as an example of the possibilities of this method.

This chapter could not be better. It does not mention however the numerous solvent extraction methods now used to concentrate traces (usually inorganic) by simple extraction with complexing agents. These methods are far removed from the work of Dr. CRAIG. Thus the reviewer feels that there is scope for another chapter dealing with these topics which was unfortunately not included in the symposium.

The only other chapter dealing with chromatography is in the last part of the symposium. In Chapter 24 R. E. THIERS gives amongst other examples the determination of Co in blood by ion exchange chromatography by means of a flow sheet.

It is felt that Chapters 2 and 3 warrant the purchase of this book for every newcomer to chromatographic methods.

M. LEDERER (Arcueil)

NEW BOOKS

Handbuch der Papierchromatographie, by I. M. HAIS AND K. MACEK.
Band I: Grundlagen und Technik. German translation of the second Czech edition by J. LIEBSTER. (VEB G. Fischer Verlag, Jena, 1958), about 900 pp.

Radioaktive Isotope in der Biochemie, by E. BRODA, with a preface by G. v. HEVESY. (Verlag Franz Deuticke, Vienna, 1958), 326 pp. with 30 illustrations, price ÖS 294.—, DM 49.—.

Literaturzusammenstellung über Papierelektrophorese, herausgegeben von Dr. Bender und Dr. Hobein GMBH, Munich, 39 pp.
Without claiming completeness this collection of references lists papers on paper electrophoresis up to Sept. 1, 1956. Titles are sometimes translated into German and sometimes left in the original.

Chromatographische Methoden in der analytischen und präparativen anorganischen Chemie unter besonderer Berücksichtigung der Ionenaustauscher, by E. BLASIUS. (Ferdinand Enke Verlag, Stuttgart, 1958), XX and 370 pages, price DM 99.—.

Gas Chromatography. A symposium held under the auspices of the analysis instrumentation division of the instrument society of America, August 1957. Edited by V. J. COATES, H. J. NOEBLES AND I. S. FAGERSON. (Academic Press, Inc., New York and London, 1958), price $ 10.

REVIEW

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STARCH ELECTROPHORESIS

I. STARCH BLOCK ELECTROPHORESIS

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INTRODUCTION

Starch is used as supporting medium in three zone electrophoretical techniques:

I. Starch block electrophoresis, introduced by KUNKEL AND SLATER[1, 2].

II. Starch column electrophoresis, developed independently by CARLSON[3] and FLODIN AND PORATH[4].

III. Starch gel electrophoresis, first described by SMITHIES[5, 6].

The name zone electrophoresis has been introduced by TISELIUS[7] in order to indicate a difference with moving boundary electrophoresis. While in the moving boundary or free electrophoresis one works in dilute solutions with the inevitable result of only partial separation of different fractions, in zone electrophoresis the application of a solid material as supporting medium allows the separation of a mixture into discrete zones.

Various materials have been used as stabilizing medium. Especially for the separation of proteins, peptides, and probably also ribonucleic acids, starch has some advantages over paper, gelatin, agar, silica, asbestos fiber, or glass powder.

(i) Adsorption of most proteins on starch is only small.

(ii) Elution of starch segments after the electrophoretic run is easier than in the case of gelatin or agar gel which is an important factor for preparative purposes.

(iii) Starch forms more easily a homogeneous paste with buffers than does cellulose or glass powder.

(iv) Electrosmosis is lower than in most other supporting media[2] although this phenomenon may cause poor separation also in the starch block technique, mainly when electrophoresis is carried out with horizontal arrangement of the starch block[8]. Starch electrophoresis is generally considered to be a rather gentle purification method for proteins, that is to say that there is no more chance of denaturation than there is in the method of salting-out or precipitation with acids, alcohol or acetone. SORKIN *et al.*[9] on the contrary claim that during the electrophoretic run irreversible denaturation processes lead to considerable loss of protein. The question arises whether this holds true for their special case or that heat development, caused by the 17–20 mA current in their experiments with 0.1 μ buffer solutions, was responsible for the observed denaturation. Buffers of lower ionic strength than 0.1 μ usually give better results.

HARRIS AND MEHL[10] found a decrease of 40–50 per cent in enzymatic activity during electrophoresis of crude intestinal alkaline phosphatase. These authors could not give an explanation for this effect but were able to show that incomplete recovery from starch would cause a loss of 12–13 per cent only.

Hereafter we shall discuss the most important applications, and our own experience with starch block electrophoresis, which has been introduced as routine technique in the Laboratory of Anatomy and Embryology ((University of Amsterdam) and in the Department of Biochemistry of the Netherlands Cancer Institute.

METHODS

1. Apparatus

Starch block electrophoresis is carried out either in semicylindrical glass troughs or in rectangular plastic boxes which can easily be constructed in every laboratory. Glass troughs can be obtained by cutting Pyrex tubing in half longitudinally[11]. In our laboratory perspex boxes 40 × 2 × 4 or 80 × 2 × 8 cm appeared to be convenient for analytical and large scale preparative work. The use of a starch block simply packed in wax paper as originally described by KUNKEL AND SLATER is not advisable. When very high voltages are required a water-jacketed box may be used[12].

2. Electrode vessels

Reversible Ag–AgCl electrodes as well as agar or filter paper bridges between the electrode vessels are sometimes used[12–14, 56]. Very simple electrode vessels are made of

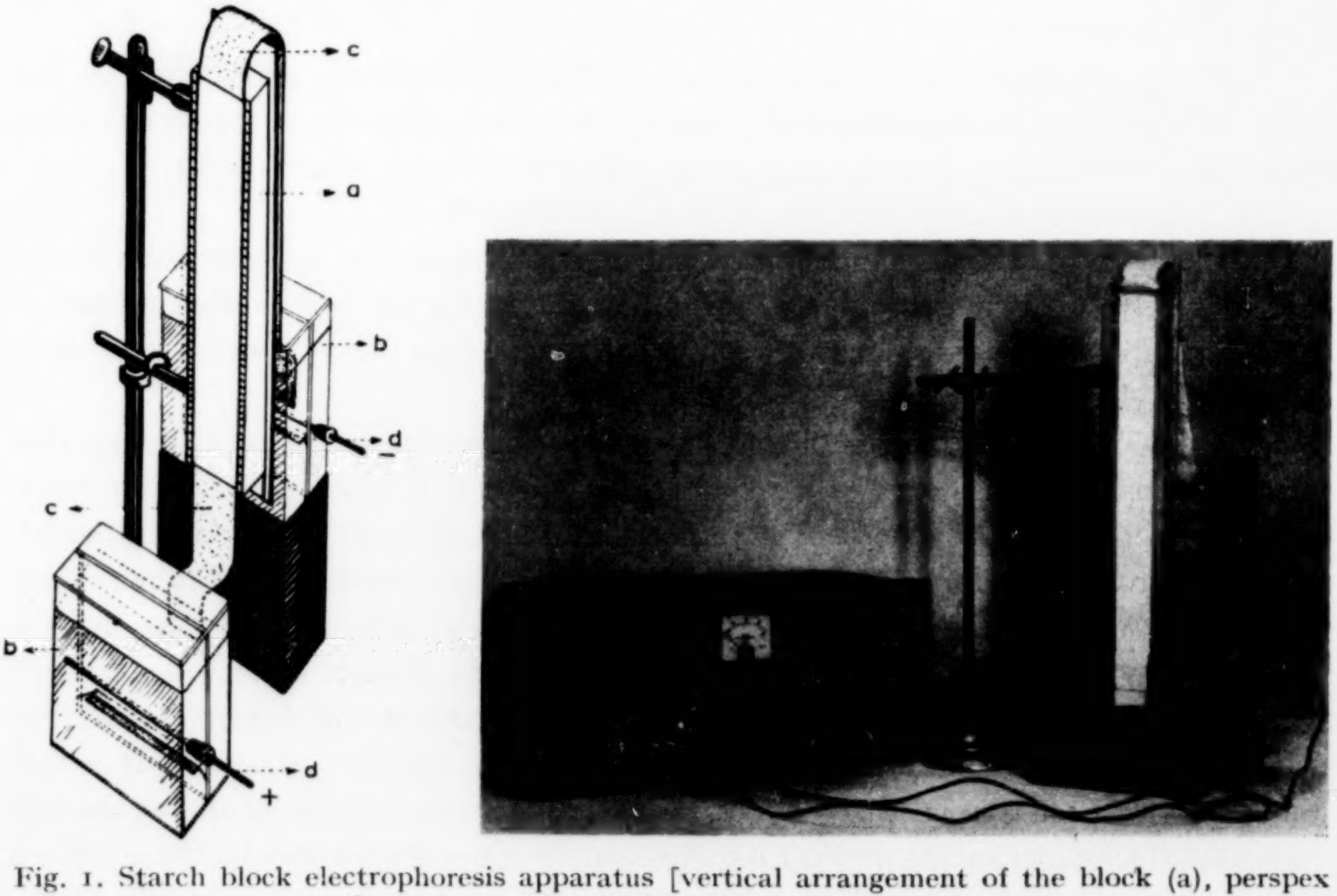


Fig. 1. Starch block electrophoresis apparatus [vertical arrangement of the block (a), perspex electrode vessels (b) with carbon electrodes (d)].

perspex 18 × 7 × 14 cm. Each vessel is divided into two compartments by means of a perspex plate (Fig. 1, b). A cotton wick pressed into a slit in this plate serves as fluid bridge. Separated compartments are necessary to maintain the pH constant in that part of the vessel which is connected with the starch block. While there is a considerable change of pH in the outer electrode compartments, there is but a very slight decrease or increase, respectively, of pH in the inner compartments of the anode and cathode vessel. When experiments are carried out for longer than 24 hours it becomes necessary to renew the buffer solution in the electrode vessels. Inexpensive carbon rods (Fig. 1, d) appear to be satisfactory in various buffers, although at higher voltages carbon anodes show a tendency to disintegrate. In these cases they may be replaced by coiled platinum wire. Balance in the electrode vessels is obtained by connecting cathode and anode with a rubber tube. This tube has to be closed during the electrophoretic run. Balancing of the levels is unnecessary when the box is arranged vertically and the cathode vessel is placed higher than the anode vessel (Fig. 1). When a series of troughs at different pH's or ionic strengths are run at the same time, the anode chamber will have to be divided in subcompartments into which the troughs dip, as described by PAIGEN[12]. This arrangement protects the troughs from any change induced by electrosmosis. For the cathode vessel this division is not required.

During the electrophoretic run the vessels are closed with perspex plates or a sheet of parafilm.

3. Preparation of starch

Potato starch is the material most commonly used in starch electrophoresis. Depending on the purpose of a given investigation the starch has to be purified more or less carefully before use. If very exact analytical results are required, the starch is washed several times with distilled water; these washings are best carried out in the following manner. The starch is mixed with water in a ratio 1:3, stirred vigorously and the particles are allowed to settle. After 30 minutes the supernatant is decanted. Insoluble impurities and the smaller starch particles are removed. After repeated washings the starch is partially dried by sucking on a large Buchner funnel. The starch then is washed two or more times with the buffer in which the electrophoretic run will be carried out. KUNKEL recommends the use of warm buffer solution[14]. After these washings and drying of the starch, it is mixed once more with buffer and stirred until a homogeneous paste is obtained which is poured into the trough. Excess fluid is removed by absorption on thick filter paper. GRANICK AND MAUZERALL[15], who isolated an enzyme which converted *d*-aminolevulinic acid to phosphobilinogen, claim that the recovery could be increased when the starch is pre-washed with 0.25% bovine albumin. In order to prevent evaporation the whole starch block may be packed in water-repellent paper or covered with a sheet of parafilm. When open troughs or boxes are used the surface of the starch may be covered with a layer of molten paraffin wax[16].

The starch block (Fig. 1, a) is connected with the outer compartments of the electrode vessels by means of filter paper strips (Fig. 1, c) folded five or more times and inserted between the outer end of the block and the perspex box. The strips are enclosed in parafilm or thin plastic sheets. The connection may also be accomplished by means of plastic sponges, moist cloth, or agar bridges.

Recently RAACKE[60] found that marked changes in the conductivity and pH take place in the starch medium. These changes, caused by the ion-exchange properties of starch can largely be avoided by washing the starch with buffer before electrophoresis.

4. *Insertion of the protein mixture*

The box, connected with the electrode vessels, is left for at least 3–4 hours in the cold room for equilibration. Experiments may also be carried out in a refrigerator.

In case the proteins migrate towards the anode, a starch segment 0.5 or 1 cm wide is cut out of the block at 5–7 cm from the cathode side of the box. When the starch is covered with paraffin a window is cut in the wax layer after equilibration in the cold room.

In order to ensure a correct insertion of the mixture under investigation the following procedure is recommended[8]. The protein solution is mixed with starch so that a paste is obtained of the same consistency as the starch block in the box. Then a steel frame with sharp edges (Fig. 2, a) and the exact proportions of a transverse segment is pressed through the window in the wax layer into the block, the starch within the frame is withdrawn and the inserted segment is pressed by means of a perspex pestle (Fig. 2, b) that fits precisely in the frame. Direct application of the protein by means of a pipette which penetrates the starch block is not advisable on account of irregular flow of the solution which often results in poor separation after the electrophoretic run. Narrow zones may be obtained by careful injection of the sample into the block by means of a hypodermic syringe fitted with a blunt needle[12]. When very small quantities of material have to be separated the solution may be sucked into a piece of Whatman 3MM paper, which has the same cross-section as the starch block. An opening is cut into the starch with a spatula and the wet paper is

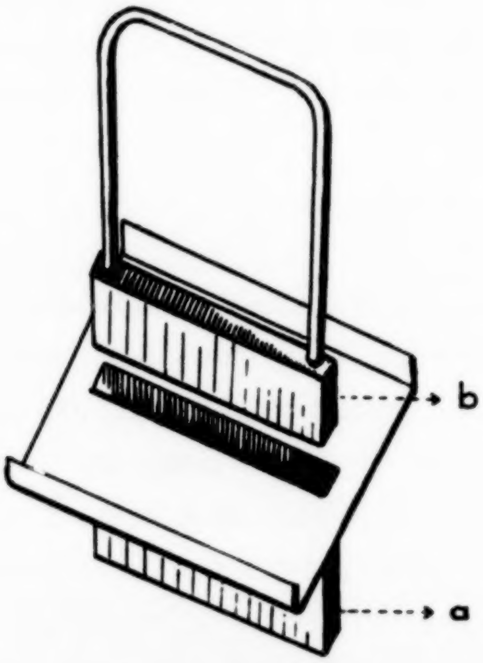


Fig. 2. Stainless steel frame for the insertion of protein-starch mixture (a). Plastic pestle with brass handle (b).

carefully put in by means of two forceps. Direct contact of the paper with the starch is obtained by slight pressure on the starch surface. In this manner tritium-labeled glycyl-alanine was separated from trace amounts of glycine and alanine[17].

5. *Electrophoresis*

The circumstances under which electrophoresis in starch medium is carried out differ from case to case. In Table I a survey is given of a number of substances studied, buffers, pH's, voltages, current, and duration of the electrophoretic run.

TABLE I

Material	*Buffer*	*Ionic strenght (μ) Molarity (M)*	*pH*	*V*	*mA*	*h*	*Ref.*
Normal and pathological serum lipoproteins	Barbital	0.1μ	8.6	100–500	25–60	24	1
	Phosphate	0.05μ	6.5				1
Serum lipoproteins	Barbital	0.1μ	8.6	400	—	24	2
Bovine lens proteins	Barbital	0.025μ	7.8	420	6–8	25	8
Tuberculo-proteins	Barbital	0.1μ	8.6	360	17–20	30	9
Intestinal alkaline phosphatase	Barbital	0.02μ	8.6	200	7–9	12	10
Growth hormone (Somatotropin)	Acetate	$0.017M$	4	175	30	24	11
	Carbonate	$0.1M$	11.2	175	30	72	11
Tobacco mosaic virus	Phosphate	$0.01M$	7.1	380	—	3¼	12
Rabbit reticulocytes	Tris	0.05μ	7.65	270	90	27	15
Chicken erythrocytes	Phosphate	0.13μ	6.9	300	80	19	15
Glycyl-alanine (Tritium-labeled)	Acetate	$0.02M$	3.9	180	4	45	17
β-Galactosidase	Pyrophosphate	$0.025M$	8.5	370	11	16¼	18
Bacterial RNA	Tris	0.015μ	7.6	5.5V/cm	—	15	19
Staphylococcal cell extracts	Tris	$0.05M$	7.6	600	—	4½	26
Chymotrypsinogen	Cacodylate	0.1μ	6.6	3.2V/cm	—	65	27
α-Corticotropin	Acetate	0.1μ	4.0; 5.2	200	—	24	28
	Cacodylate	0.1μ	6.6; 7		—		28
	Barbital	0.1μ	8.3		—		28
Inorganic anions	Acetate	—	7	300	5–15	1	29
Inorganic cations	Lactate	—	3.5; 6.5	800	10–30	½	29
	Phthalate	—	4.5				29
Thyroid stimulating hormone	Phosphate	0.1μ	7.4	300–600	30–50	16–22	31
^{14}C-labeled plasma protein	Barbital	—	—	400–500	24–32	24–36	37
Diphteria antitoxin	Barbital	0.1μ	8.6	500	50–60	18–20	38, 39
Follicle stimulating hormone	Acetate	0.1μ	4	440	10	20	40
Albumin, α-globulin	Barbital	—	8.6	112	27	36	43
Prolactine S. acetamide	Carbonate	$0.1M$	11.2	3.5V/cm	—	48	46
Mammalian tyrosinase	Barbital	0.1μ	8.5	300	11	19	48
Trypsin	Acetate	$0.1M$	4	300	—	10	49
Ribonucleoproteins	Phosphate	0.1μ	9	350–435	—	5¾	50
	Phosphate	0.08μ	8.2	150	—	12	50
Proteins from microsomes	Phosphate	$1.5M$	7	225	11–13	12	53
Proteins from mitochondria	Phosphate	$0.05M$	7.4	6V/cm	20	16–18	55

6. *Influence of temperature*

When heat-labile substances are under investigation effective cooling often becomes a serious problem. Control experiments carried out in our laboratory showed a heat effect as indicated in Fig. 3. Working in a cold room without further precautions is not sufficient in itself. The rise of temperature, illustrated in Fig. 3, may be restricted to 3° if the temperature of the cold room is kept at —4° during electrophoresis. According to PAIGEN[12] 10 watt over a 24 hours period may be applied if the block is cooled with circulating ice water. The use of a water-jacketed box is then necessary. ROTMAN AND SPIEGELMAN[18] obtained efficient cooling by having the trough in contact both from above and below with lead bricks such as are commonly used for radioactive shielding.

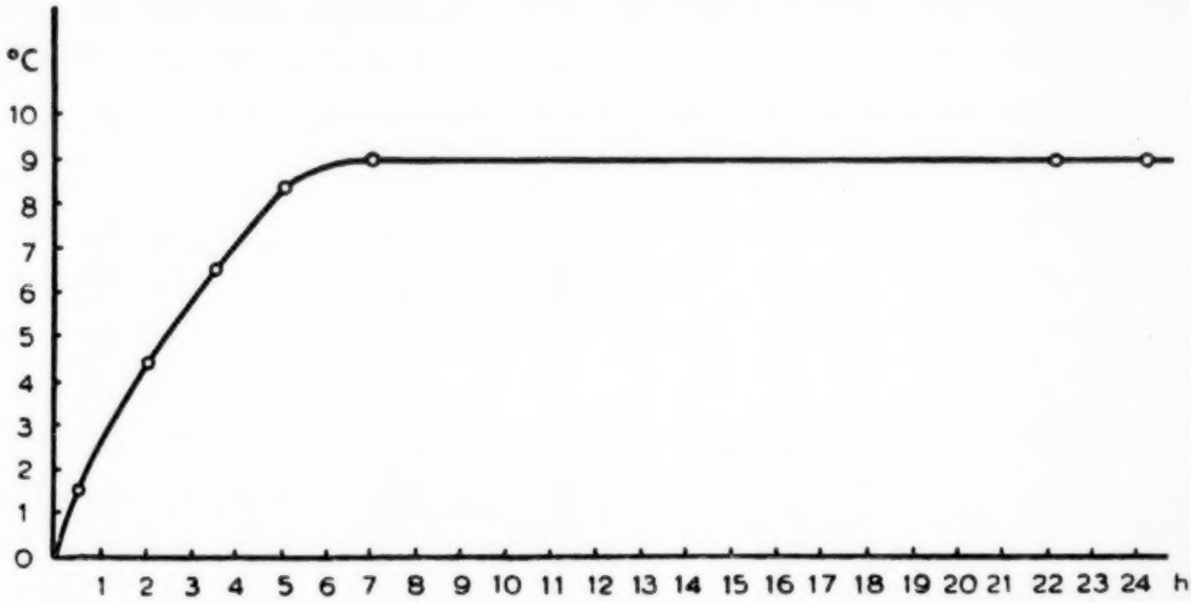


Fig. 3. Rise of temperature in a 40 × 2 × 4 cm starch block during electrophoresis at 200 V, 6–8 mA; in phosphate buffer 0.025 μ. Temperature of the cold room: 2° C.

7. *Location of substance*

After the electrophoretic run, the paraffin layer, the parafilm or perspex cover is carefully removed and a strip of Whatman paper is pressed against the starch surface. It is dried for 5 minutes at 80–100°. In case starch particles adhere to the strip they are removed with a soft brush. The strip then is coloured with Amido Black or any other protein dye. In this manner one is able to find the approximate position of protein components which is important mainly for preparative work when sectioning of the whole block is not necessary. The position of nucleic acids is determined by observation of a "print" on Whatman 3MM filter paper under ultraviolet light at 260 mμ[19]; protein, however, will interfere. Radioactive substances may be detected by examination of the paper under a strip counter. Examination of the starch block under ultraviolet light may also be useful when the material shows fluorescence. The most exact location of components is realized by cutting the block, transferring the starch segments to centrifuge tubes, followed by elution and protein estimation in the supernatant after short centrifugation. Settling of the starch without centrifugation is not advisable on account of possible interference of very small particles with protein or RNA determination. The run of sera can be followed by mixing them with a quantity of stain which is bound to the albumin fraction[16].

8. *Cutting*

CARLSON[3] already noted that it is difficult to cut the starch with precision. According to our experience errors of 10–20% may arise when segments of 0.5 cm are cut in the way originally described by KUNKEL AND SLATER[2]. The method of PAIGEN[12] too gives results of insufficient exactness when proteins, the mobilities of which differ only little, are under investigation.

In order to circumvent these troubles a calibrated box permitting more exact cutting was originally used by us[8]. A guillotine-like frame (Fig. 4, a) is moved over this box. The frame holds a stainless steel knife with the proportions of the cross-section of the starch block (Fig. 4, b). Although this method improves the slicing technique considerably, small distortions of the starch block may occur which result in poorly reproducible patterns after protein estimation. Furthermore, this procedure is time-consuming, particularly when long boxes are used. Substantial improvement is obtained with the apparatus shown in Figs. 5A and 5B.

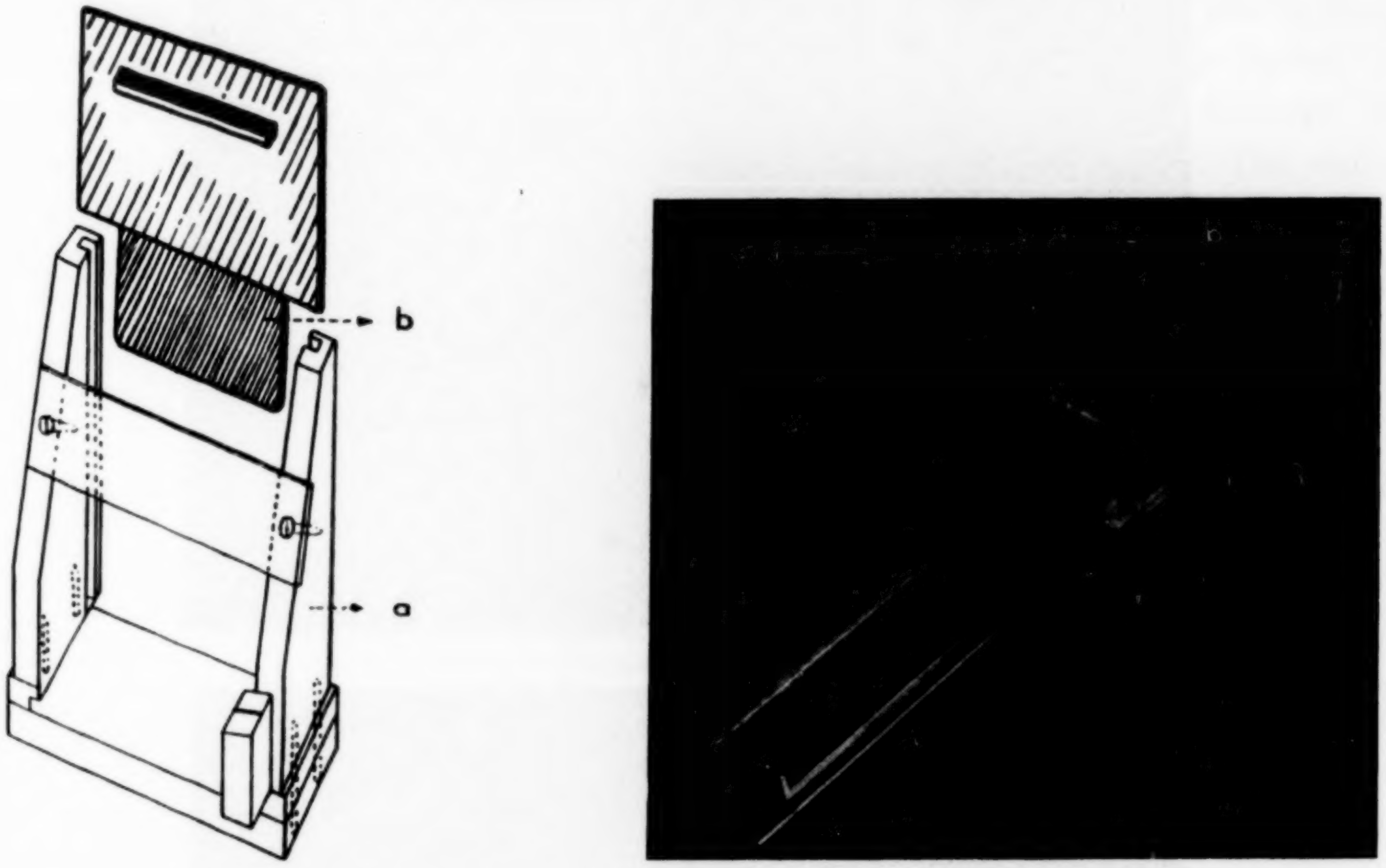


Fig. 4. Cutting device for starch block electrophoresis. (a) Perspex frame. (b) Stainless steel knife.

A perspex box with removable side walls (Fig. 5, a) fits in a cutting device illustrated in Fig. 5, b. The latter consists of a steel frame joined to a metal plate by means of hinges. Thin stainless steel threads are spanned on the frame at 1 cm intervals[20].

After an electrophoretic experiment is finished the two side walls of the box are removed and the remaining bottom of the box with the starch block is placed in a fixed position on the metal plate of the cutting apparatus. The frame is turned down with the aid of handles (Fig. 5, c). The bottom of the box is supplied with notches at 1 cm intervals into which the threads fit. Cutting of the block is performed in one single manipulation and exactly equal starch segments are obtained.

9. *Elution, protein and nucleic acid determination*

The separate starch segments are eluted with ice cold water or buffer solution by stirring and centrifugation during 10 minutes. To obtain optimal recovery the following method is recommended. Each starch segment is put on a narrow-stemmed glass filter. Buffer solution added above the packed starch displaces the fluid in the segments by slight suction[14, 37]. It has, however, to be considered beforehand whether the protein will not be denatured on the filter surface. The eluates can be concentrated by

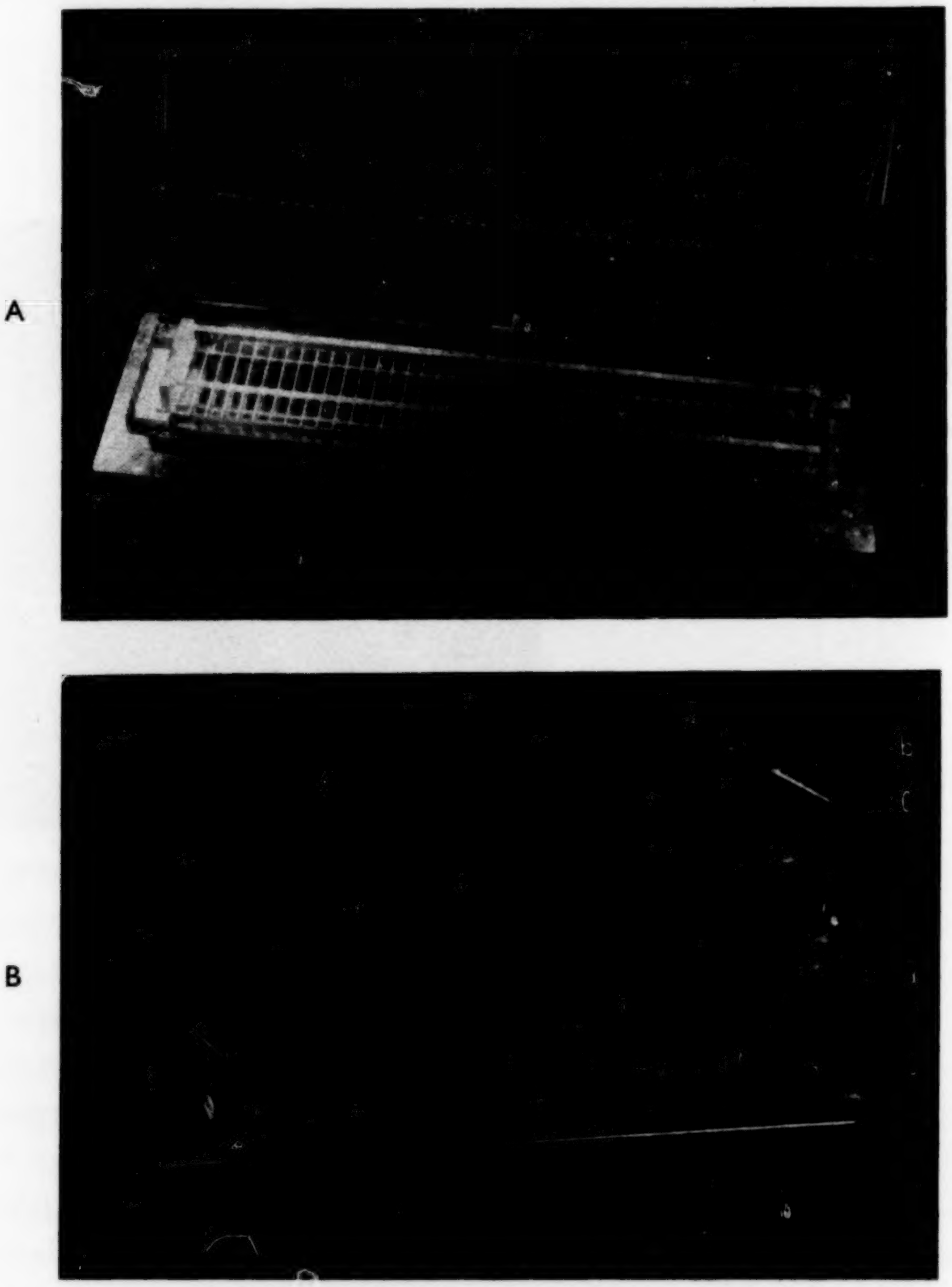


Fig. 5. [A]. Improved cutting apparatus for starch block electrophoresis. [B]. (a) Calibrated box with removable side walls. (b) Cutting device. (c) Handles.

dialyzing against dextran solution or polyvinyl pyrrolidone[21] or by freeze drying. An aliquot of the supernatant fluid is taken from each tube and the protein content is determined according to the method of FOLIN as modified by LOWRY *et al.*[22]. Also direct measurement of the protein extinction at 280 mμ is possible if the solution is completely clear. Direct measurement of nucleic acid contents may be carried out at 260 mμ, but protein or starch traces interfere. Ribonucleic acid and desoxyribonucleic acid are conveniently estimated according to the orcinol[23] and indole[24] methods, respectively. In the case of radioactive material samples of the eluate may be counted in a Geiger-Müller or Flow counter[25, 26]. (For hormone and enzymatic activity measurements compare the references discussed in the paragraph dealing with applications, p. 131.)

10. Influence of electrosmosis

In some experiments poor separations are obtained due to a horizontal arrangement of the starch block, or when the buffer solution levels in the electrode vessels are of equal height. Under these circumstances the electrosmotic flow towards the cathode counteracts the electrophoretic migration towards the anode. The starch acquires a negative charge in relation to the buffer, since it is the stationary phase and the buffer moves towards the negative electrode. Dextran, a well known substance for estimating the degree of electrosmosis in paper[13] is not conveniently detected in starch[27]. Table II shows the electrosmotic flow for various media expressed in terms of mobility and in relation to the distance of migration of albumin (in barbital buffer 0.1 μ; pH 8.6) as observed by KUNKEL AND SLATER[2]. It appears that electrophoresis on potato starch (with the exception of filter paper) undergoes the smallest influence of the electrosmosis phenomenon.

TABLE II

Medium	$\mu_{el} \times 10^5$	d_{el}/d_{alb}
Filter paper (Whatman 3 MM)	1.5	0.30
Potato starch	2.5	0.62
Washed sea sand	4.3	1.95
Ground glass (35– 60 mesh)	5.1	3.64
Ground glass (150–200 mesh)	5.9	9.87
Soft glass bead (200 mesh)	5.6	6.20
1% Agar	4.7	2.60

For the determination of electrophoretic mobilities, μ_{el}, in starch it is necessary to know the displacement of the protein due to electrosmosis, d_{el}[28]. One method to study this is running two known proteins in free as well as in starch electrophoresis; d_{el} then may be calculated from equation (1)

$$\frac{\mu_1}{\mu_2} = \frac{d_1 - d_{el}}{d_2 - d_{el}} \tag{1}$$

wherein

μ_1 = the mobility of protein 1 in free electrophoresis at the same pH applied in starch electrophoresis,

μ_2 = the mobility of protein 2 in free electrophoresis at the same pH applied in starch electrophoresis,

d_1 = the distance over which protein 1 migrates in the starch block,

d_2 = the distance over which protein 2 migrates in the starch block (under identical conditions of time and field strengths).

μ_{el} may be computed from the following equations (2) and (3):

$$F = \frac{V}{l} \tag{2}$$

$$\mu_{el} = \frac{d_{el} \cdot l}{Vt} = \frac{d_{el}}{Ft} \tag{3}$$

wherein

l = length of trough (cm), F = field strength, V = average voltage across the ends of the box, t = time (sec).

When the isoelectric point of a protein has to be determined, another calculation may be carried out. The unknown protein is subjected to an electrophoretic run, side by side with a protein of known isoelectric point. In this case equation (4) is used:

$$P_x = P_x\,(\text{obs}) - (P_i\,(\text{obs}) - P_i) \tag{4}$$

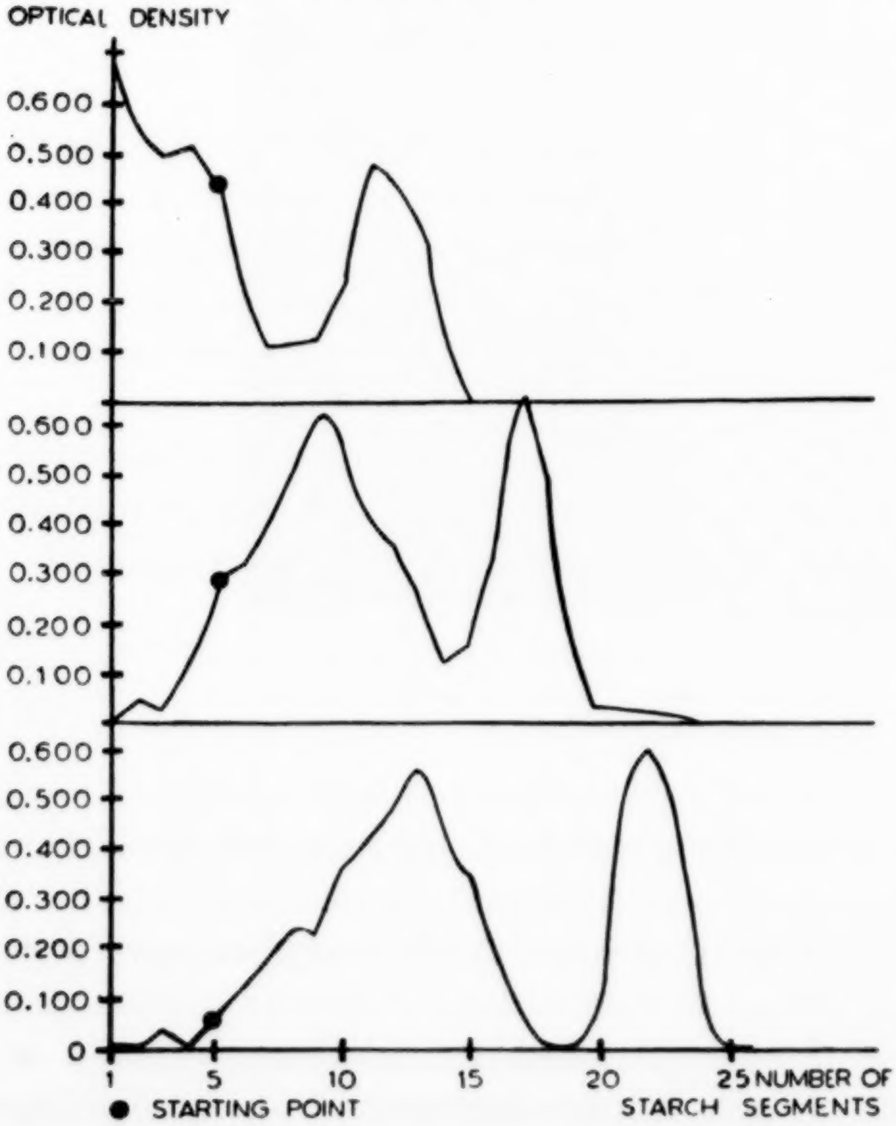
wherein

P_x = the unknown isoelectric point,

P_i = the isoelectric point of the known protein,

P_x(obs) and P_i(obs) = isoelectric points of unknown and known protein, respectively, obtained by plotting the distance of migration against pH.

Fig. 6 demonstrates the effect of electrosmosis on the separation of the water-soluble proteins of the bovine eye lens[8].



(a) Horizontal arrangement of the starch block; buffer vessels at equal height.

(b) Horizontal arrangement of the starch block; difference in levels of buffer = 20 cm.

(c) Vertical arrangement of the starch block; difference in levels of buffer = 20 cm.

Fig. 6. The influence of electrosmosis on the separation of water-soluble lens proteins.

APPLICATIONS

As appears from Table I starch block electrophoresis is applied for the separation of various proteins, hormones, enzymes and nucleic acids. Furthermore, attempts have been made to submit inorganic ions to this separation technique[29]. It seems, however, that until now paper or column chromatography give better results for inorganic ions. In addition to the separation and isolation of certain compounds from mixtures the method is repeatedly used as a homogeneity criterion.

(*a*) *Proteins and protein hormones*. The α and β-lipoproteins were readily separated and could be followed by phospholipid and cholesterol analysis on the starch segments. No success was achieved in an attempt at direct staining. Further subdivision of these lipoproteins was obtained with low ionic strength buffers[1,2].

BLOEMENDAL[8] separated α-crystalline from the other water-soluble proteins. This protein migrated as a single boundary when subjected to free electrophoresis at different pH's above and below its isoelectric point[30].

FØNSS-BECH AND LI[11] studied the behavior of somatotropin isolated from the anterior lobe of ox pituitaries. These authors showed that their preparation migrated as a single zone at different pH's. Furthermore, they were able to estimate the isoelectric point.

RAACKE AND LI[28] described the determination of corrected electrophoretic mobilities of different proteins. In their procedure the isoelectric point of corticotropin was found to be 6.6 in monovalent buffers.

POSTEL[31] fractionated serum and pituitary preparations. The uptake of ^{131}I by the chick thyroid gland was used as indicator of TSH activity. The thyroid-stimulating activity of a commercial "pure" TSH preparation appeared to be associated with the protein peak, and was transported primarily with the γ-globulins of human serum when added *in vitro*.

KUNKEL AND WALLENIUS[32] succeeded in showing a new hemoglobin in normal adult blood. This component did not appear clearly in free electrophoresis.

GERALD, COOK AND DIAMOND[33] identified normal oxyhemoglobin and hemoglobin M in a hemolysate obtained from a patient affected with methemoglobinemia.

KUNKEL, TAYLOR AND DU VIGNEAUD[34] were able to carry out a biological assay of an oxytocin fraction isolated after starch block electrophoresis.

MÜLLER-EBERHARD AND KUNKEL[35,36] described a subfractionation of human γ-globulin. The experiments were partially performed in polyvinylchloride as supporting medium. This material is more suitable for carbohydrate analysis, as starch is less satisfactory on account of contamination of carbohydrate derived from the medium.

MILLER AND BALE[37] applied the starch block technique in order to separate ^{14}C-labeled plasma proteins produced by the normal rat and by the isolated perfused liver. The latter incorporated radioactive lysine into the plasma albumin, α-globulin, β-globulin and fibrinogen.

KUHNS[38,39] separated precipitating and non-precipitating skin sensitizing diphteria antitoxin.

Concentration of FSH activity was achieved by means of starch electrophoresis[40]. Two fractions were obtained representing 42 and 23%, respectively, of the total protein applied. According to the author the low recovery was due to losses of dialyzable material in one of the fractions.

The utility of the described method as purification technique in combination with other isolation procedures was shown by LI and coworkers[41]. They succeeded in obtaining a peptide with high ACTH activity from sheep pituitary. The whole isolation and purification scheme involved: dioxan fractionation, starch electrophoresis, column chromatography and counter current distribution. The same laboratory reported the purification of interstitial cell-stimulating hormone[42]. This hormone was subjected to saline extraction, ethanol precipitation, ammonium sulphate fractionation, column chromatography and zone electrophoresis on starch.

LARSON *et al.*[25] identified thyroxin radiochromatographically in a globulin fraction from serum which was separated by means of zone electrophoresis on starch.

GOLDSWORTHY AND VOLWILER[43] studied the mechanism of protein turnover with labeled plasma proteins of the dog. The starch block technique was applied in combination with the COHN fractionation method[44, 45] in order to obtain γ-globulin, fibrinogen, α-globulin and albumin.

Preparations of reduced lactogenic hormone when submitted to zone electrophoresis on starch[46] showed only one peak. The mobility of the reduced hormone appeared to be higher than that of the native prolactin.

SORKIN *et al.*[9] applied a zone transfer technique from starch into glass powder. These investigators are among the few to obtain poor yields with starch block electrophoresis.

(*b*) *Enzymes.* WETTER[47] fractionated proteases from *Mortierella renispora* Dixon-Stewart. Nearly quantitative recovery was obtained for both the activity and nitrogen content. When the separation was performed by means of Whatman No. 3 paper, the yields were extremely poor.

HARRIS AND MEHL[10] achieved approximately 260-fold purification of a crude preparation of alkaline phosphatase from bovine intestinal mucosa. However, the amounts of purified product were only small. The results of these authors indicate that the enzyme may have been present initially in a complexed form and that this complex was disrupted during sequential electrophoretic runs in several starch blocks.

Efforts to remove protein impurities present in chromatographically prepared mammalian tyrosinase did not result in further purification. Approximately 99% of the activity was recovered[48].

LIENER AND VISWANATA[49] separated autolysed trypsine into an active fast moving component and a less active fraction which corresponded to dialyzable split products.

ROTMAN AND SPIEGELMAN[18] investigated extracts of *Escherichia coli* for β-galactosidase activity. One single run could effect a 30-fold purification whereas 80% of the available enzyme could be recovered.

(*c*) *Ribonucleoproteins and nucleic acids.* ELSON[50] subjected ribonucleoproteins to zone electrophoresis on starch before and after treatment with urea. According to his findings there are indications that hydrogen bonds play an important role in the linkage of RNA with the protein.

PAIGEN[12] applied the method to tobacco mosaic virus strains. Separation into three components was possible.

PARDEE, PAIGEN AND PRESTIDGE[19] studied the electrophoretic behavior of RNA, DNA and protein from *Escherichia coli* and *Bacillus megatheria*. They were able to show that bacterial RNA migrates only in two distincts zone. The electrophoretic patterns obtained depended on the buffers employed, a phenomenon which is also well known in paper electrophoresis[51, 52]. Approximately 90% of the nucleic acid and 70% of the protein were recovered.

(*d*) *Proteins from cell fractions.* Lately, starch block electrophoresis is being used also for the study of subcellular fractions. GRANICK AND MAUZERALL[15] described the fractionation of a soluble enzyme fraction from red cells. They observed one main component migrating towards the cathode, which appeared to be hemoglobin, whereas three colorless enzyme fractions moved towards the positive electrode. A total of 30 ml fluid could be fractionated.

ARCOS AND ARCOS[53] made an attempt at detecting structural alterations in liver microsomes during chemical carcinogenesis. Swelling had been used to detect these alterations[54]. The swelling of microsomes from liver tissue was paralelled by the release of a group of soluble proteins not sedimenting at 105,000 *g*. These proteins were resolved into two fractions on starch. Microsomes from hepatoma on the contrary showed low swelling with relatively high protein release. These investigators did not include the proteins of hepatoma in their electrophoretic studies.

WIRTZ AND ARCOS[61] made a comparative study of the electrophoretic pattern of rat liver and hepatoma supernatant fluids. The so-called protein "h" was isolated and its molecular weight, amino acid composition, and bound azo-dye were determined.

BERESOVSKAYA[55] isolated an enzyme from mitochondria which catalyzed the amino acid synthesis from pyruvic acid and ammonia. The active protein was present in a fast migrating fraction which had a higher mobility than had serum albumin. The electrophoretic separation resulted in a 300–350 fold purification of the enzyme as compared with the original liver homogenate.

GALE and coworkers[26] isolated a fast moving fraction from extracts of disrupted staphylococcal cells which were previously incubated with ^{14}C-L-glutamic acid as the only labeled amino acid precursor. The authors provide evidence that this fraction represents an intermediate stage in protein synthesis. If this is so the L-glutamic acid containing intermediate should be very stable as the heat development in a starch block under the conditions of their experiment (20 V per cm, pH 7.6) may be considerable.

BLOEMENDAL AND BOSCH[62] fractionated the ^{14}C-leucine labeled pH5 enzyme from rat liver cytoplasma. An important working condition was air-cooling of the starch block to —5°.

(*e*) *Homogeneity studies.* RAACKE[27,28,56] used starch electrophoresis for homogeneity studies of ribonuclease, lysozyme, bovine serum albumin, chymotrypsinogen and chymotrypsin. All preparations investigated yielded more than one peak after variation of buffers and the application of a pH-range from 4.7 to 9.1. These results emphasized once more the importance of pH variation. On the other hand one has to realize whether the peaks observed are a proof of heterogeneity or that the substance under investigation was no longer stable at any "extreme" pH in the alkaline or acid range. Such stability boundaries were observed *e.g.* in the case of the lens protein α-crystalline below pH 4 and above pH 8.9[57]. According to RAACKE starch electrophoretical homogeneity experiments may be considered to be as sensitive as the reversible boundary spreading test[58] and superior to the moving boundary method.

WADA *et al.*[59] concluded to the homogeneity of soy bean hemagglutinin after finding one peak at one pH. In this connection it must be mentioned that a number of workers are as yet satisfied with one single pH value. The conclusion reached then can only be that the protein is *electrophoretically* homogeneous at *this* pH.

CONCLUSION

Starch block electrophoresis is of growing importance as a useful tool in protein chemistry for analytical studies as well as for isolation on preparative scale. However, reproducible results will be obtained only if the working methods are exactly standardized. In a few cases polyvinylchloride as a supporting medium may be more satisfactory than starch; working conditions, however, remain identical.

Poor separation may sometimes be the result of horizontal arrangement of the troughs. Gravity then may cause accumulation of protein in the lower starch layers, leading to uneven mobilities at different levels of the block. Furthermore, the electrosmotic flow may counteract the electrophoretic migration. Vertical position of the starch block is therefore preferable.

In summary, the starch block technique opens up the possibility for separation of various protein mixtures; it is often successfully applied in combination with other isolation methods and, in some cases, gives good results where other fractionation procedures fail.

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DETECTOR FOR LIQUID-SOLID CHROMATOGRAPHY

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INTRODUCTION

The need for a means of indicating or recording changes in the composition of liquids flowing from liquid–solid chromatographic columns is long-standing. Methods that have been used include the continuous measurement of refractive index[1], conductivity[2,3], dielectric constant[4], pH[5] or absorption of light of suitable wavelength[6]. These methods often involve costly and complicated equipment and are highly sensitive only when liquids having very different physical properties are involved.

The detector described in this report depends upon the differences in the heat of adsorption of liquids on suitable adsorbents. The liquid leaving the chromatographic column is passed through a tube containing the adsorbent in which a thermocouple is embedded. As the composition of the liquid passing through the detector changes, the temperature of the adsorbent and the thermocouple changes. These temperature changes are a function of the heats of adsorption and desorption of the components of the liquid passing through the detector.

After passing through the detector the liquid may be collected in fractions for further examination and signals from the thermocouple may be used to operate a fraction collector.

The detector is simple and easily constructed in the laboratory and has a fairly high sensitivity. It is suitable for the detection of the different classes of hydrocarbons that occur in benzole, petroleum and similar complex mixtures and can also be used for the detection of metals in eluates obtained from chromatography on ion-exchange resins.

THEORETICAL CONSIDERATIONS

The adsoprtion of gases and liquids on surfaces of solids is accompanied by the evolution of heat due to a decrease in free energy of the system. This heat is called the heat of adsorption. The evolution of heat that occurs in the detector described in this report is due to the replacement of one substance adsorbed on the solid by another. The replacement may be of air by a liquid, of one liquid by another, or of a liquid by a solute dissolved in the same liquid.

The most important factors influencing the amount of heat evolved in the detector are:

1. The nature of the material being displaced from the adsorbent.
2. The nature of the displacer.

3. The quantity of solid adsorbent in the detector.
4. The adsorbent capacity and nature of the adsorbent.
5. The quantity of material replacing that originally on the adsorbent.

If the quantity of adsorbent in the detector is small compared with the quantity of any one component in the liquid leaving the column, the heat evolved when a new component reaches the detector will be a characteristic of the compound concerned but will be independent of the quantity of the compound present. Fig. 1 shows this type of detector. It is the temperature change and not the heat evolved that is measured in the detector, and the following will affect the temperature change:

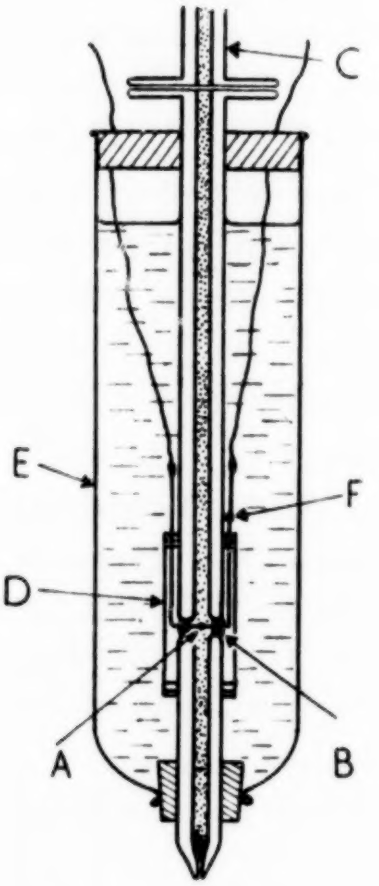

Fig. 1. The detector.

a. Thermal capacity of the body of the detector and the thermocouple.

b. Specific heat of the liquid in the detector.

c. Quantity of liquid in the detector.

d. Heat losses from the detector by conduction away from the detector and normal heat losses to the surroundings.

e. Rate of flow of the liquid through the column.

For a detector intended only to indicate changes in the composition of the liquid leaving the column, these factors are of little importance.

DESIGN AND OPERATION OF APPARATUS

The detector, shown in Fig. 1, indicates changes in the composition of the liquid that passes into it. It is attached to the bottom of the chromatographic column C by a flat-flange or taper joint, which is suitably lubricated and clamped to be leak-proof.

The detector consists of a length of capillary tubing, 1.6 mm I.D., carrying a thermojunction A so placed that the junction is in the centre of the bore of the capillary. The thermocouple wires pass through the holes B and are sealed in place with litharge–glycerin cement. The tube D is fitted on to the capillary tube with rubber bungs so that the part of the capillary near the thermojunction is enclosed in an air jacket. The wires pass through the upper rubber bung and the cold junction F is made just above this bung. The detector is surrounded by a tube E containing water. This water jacket keeps the walls of the detector at a constant temperature, thus ensuring a uniform rate of heat loss. It also keeps the cold junction F at a constant temperature. The lower end of the capillary tube is drawn out to a fine jet and a few coarse granules of silica gel are placed in the jet to retain the adsorbent. Suitable thermocouples may be made from wires, 0.004 in. in diameter, of 40% palladium in gold and 10% iridium in platinum. The thermojunctions are best made by carbon-arc welding.

This detector may be used to indicate any changes in composition of liquids supplied to it. Its use in conjunction with displacement development is one of its more obvious applications. The dimensions of the detector described are such that it can be attached to the column used for the analysis of hydrocarbon types by the Fluorescent Indicator Adsorption (FIA) Method (I.P. 156/58T)[7].

The detector is fitted by means of flat flanges or taper joints to the lower end of the analyser section of the FIA column, the detector and the column being filled with silica gel in the normal way. The sample is introduced on to the silica gel, followed by the displacer, isopropyl alcohol. The bands corresponding to saturated, unsaturated and aromatic hydrocarbons develop and as each boundary reaches the detector the heat generated raises the temperature of the thermocouple. The thermocouple cools

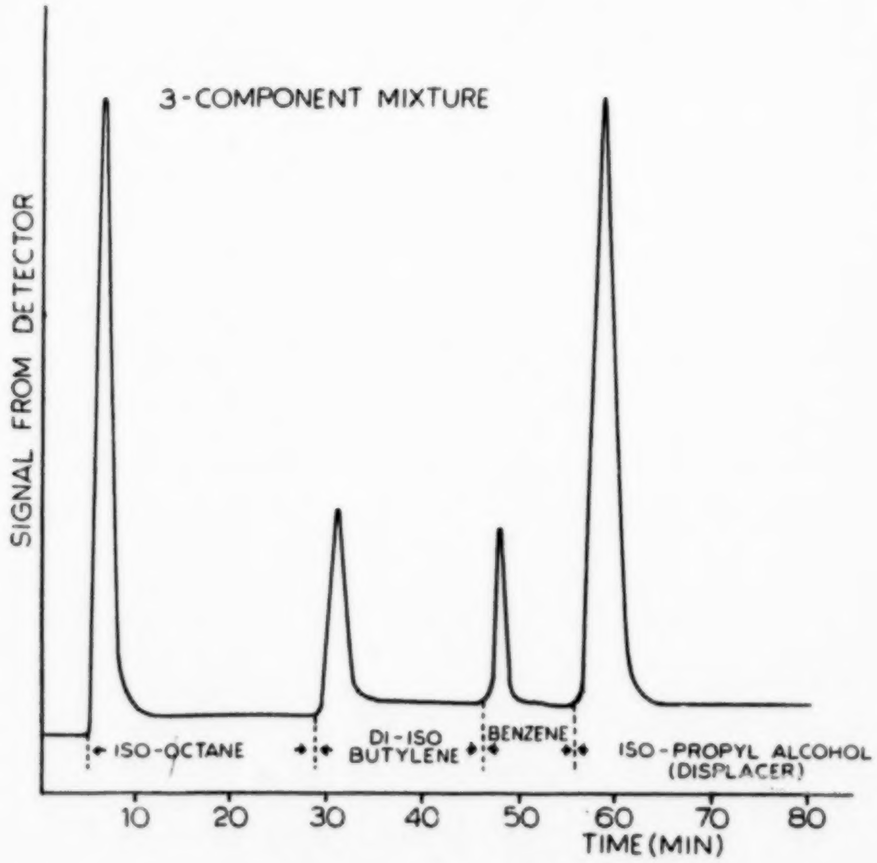

Fig. 2. Displacement chromatography.

References p. 139.

again after the boundary has passed it. The output from the thermocouple is supplied to a suitable galvanometer or recorder. On larger columns the output may be used to operate a fraction changer, thus enabling the various types of hydrocarbons to be collected separately. Fig. 2 shows the results obtained on a mixture of iso-octane, di-isobutylene and benzene. The boundaries are quite sharp. The time axis does not provide an indication of the quantities of the hydrocarbon types present, due to variations in flow rate, which was not accurately controlled.

DISCUSSION AND CONCLUSIONS

The qualitative detector is a simple device that will give a signal when a boundary reaches it during a chromatographic separation. As it is merely a thermocouple inserted in the column, it can obviously be used in any system in which temperature changes occur at the boundary between components. In addition to liquid–solid adsorption chromatography, it has been found to be applicable to separations carried out on ion-exchange resins, the detector being filled with the resin.

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The detector described in this paper was devised by A. GROSZEK, who has now left the service of the Company, and additional experimental work was carried out by E. ORMEROD. A patent application covering this detector has been made by Benzole Producers Limited.

The author wishes to thank the Board of Benzole Producers Limited for permission to publish this paper.

SUMMARY

A detector is described that is suitable for indicating changes in the composition of liquids flowing from chromatographic columns.

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EIN FRAKTIONSSAMMLER GERINGER STÖRANFÄLLIGKEIT FÜR DIE PRÄPARATIVE SÄULENCHROMATOGRAPHIE

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ALLGEMEINES

Bei der Anwendung säulenchromatographischer Methoden in der präparativen Chemie ist eine Anordnung, die selbsttätig die aus der Säule ablaufende Lösung auffängt und in bestimmte Fraktionen teilt, unbedingt erforderlich. Es sind deshalb heute eine Reihe derartiger Geräte im Handel erhältlich, bzw. in der Literatur bekannt geworden. Eine ausgezeichnete Übersicht nebst einer eingehenden Beschreibung der angewendeten Methoden und deren Ergebnissen findet sich bei GRUBHOFER UND LWOWSKI[1]. Dennoch seien die wichtigsten Punkte hier noch einmal kurz herausgegriffen. Die allgemeinen Anforderungen an einen Fraktionssammler sind etwa folgende:

(a) Grösstmögliche Betriebssicherheit bei gleichzeitiger Einfachheit des Gerätes um die Kosten in erträglichen Grenzen zu halten. Über die Forderung der genannten Autoren hinaus, haben wir versucht, bei einer dennoch auftretenden Störung jeden Substanzverlust zu verhindern.

(b) Konstanz der einzelnen Fraktionen. Für präparatives Arbeiten wird man allerdings grössere Toleranzen zulassen dürfen als bei Verwendung für analytische Zwecke. Wir haben daher auf diesen Punkt geringeren Wert gelegt.

(c) Vielseitigkeit und gleichzeitig einfache Handhabung. Auf diese Forderung wird später näher eingegangen.

Die im Handel erhältlichen Geräte erfüllen nicht immer alle Forderungen wenn sie billig sind, bzw. sie sind sehr teuer, wenn sie betriebssicher und vielseitig sind. Aus diesem Grund haben wir uns entschlossen, ein geeignetes Gerät selbst zu entwickeln und das Ergebnis zu veröffentlichen.

BESCHREIBUNG DES GERÄTES

Der Fraktionssammler besteht grundsätzlich aus folgenden Teilen:

(A). dem Steuergerät;

(B). dem Mess- und Sammelgefäss mit der Volumendosierung;

(C). der Auffang- und Transporteinrichtung;

(D). der eigentlichen Säule mit einem Sicherheitsventil und eventuell einer Vorrichtung zur Niveaugleichhaltung.

Literatur S. 147.

A. Das Steuergerät

Grundsätzlich lassen sich hier drei Teile unterscheiden (Fig. 6). Ein Netzgerät dient der Stromversorgung, ein Zeitschaltgerät, welches nach dem Ladeprinzip aufgebaut ist, gestattet die Einstellung der gewünschten Sammelzeiten und ein Schrittschaltwerk stellt jeweils die Verbindung zwischen dem Steuerteil und den entsprechenden Betätigungsorganen her.

Das Netzgerät liefert einerseits die Heizspannungen für die Elektronenröhren, anderseits die Anodengleichspannung und eine Gleichspannung für den Motor, verschiedene Relais und Ventile und das Schrittschaltwerk (handelsüblicher Telefon-Drehwähler mit 3 Kontaktbahnen und jeweils 11 Kontakten). Um bei Netzausfall ein Aussertrittkommen des Gerätes zu verhindern, erfolgt die Einschaltung über ein Relais mit Selbsthaltekontakt (Rel N), welches bei Stromausfall den gesamten elektrischen Teil vom Netz abschaltet. Auf eine besondere Stabilisierung der Anodenspannung wurde verzichtet, obwohl dadurch die Schaltzeiten eine etwas grössere Toleranz aufweisen. Dieser Nachteil fällt nach unseren Erfahrungen jedoch kaum ins Gewicht.

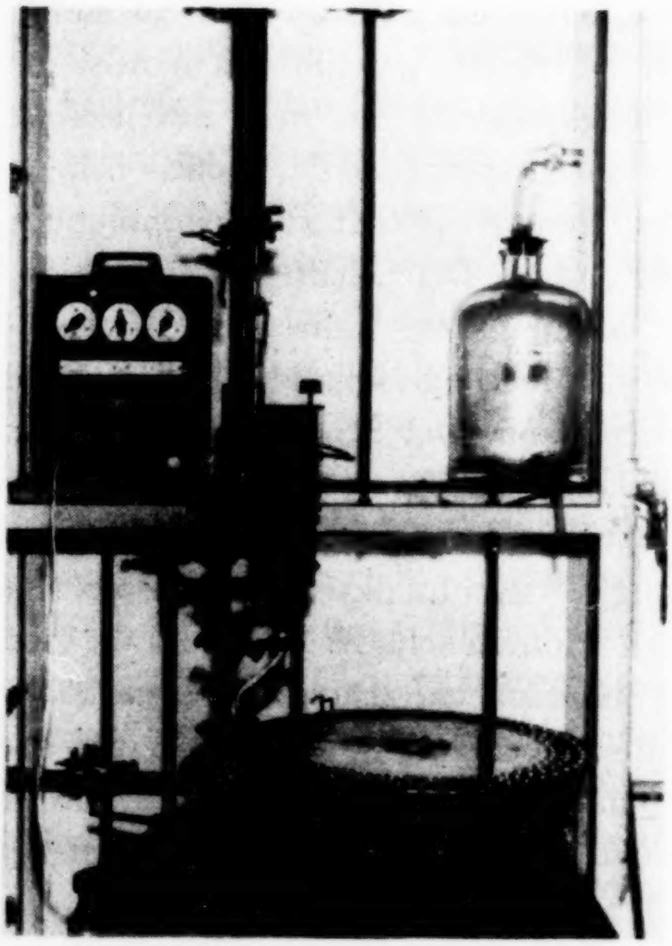

Fig. 1. Gesamtansicht des Fraktionssammlers.

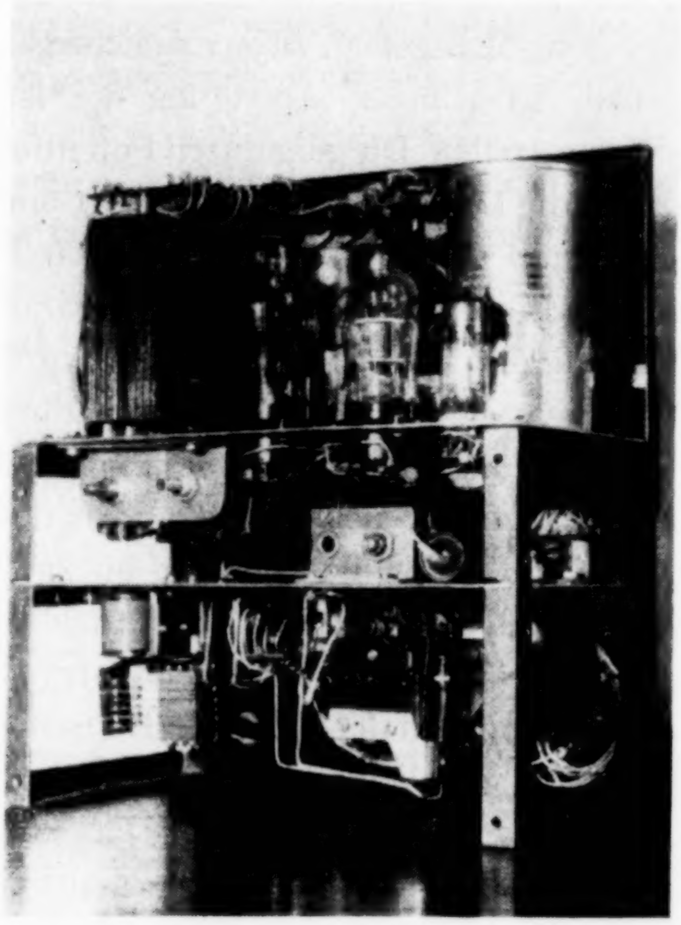

Fig. 2. Innenansicht des Steuerteiles von hinten, Gehäuse entfernt.

Das Zeitschaltgerät ist, wie eingangs erwähnt, auf dem Ladeprinzip aufgebaut. Über die Röhre EF80 wird ein Elektrolytkondensator C_L (500 μF, 500 V) aufgeladen, der nach Erreichen der Zündspannung einer Gastriode AC50 über diese schnell entladen wird. Der Entladestrom erregt das Relais Rel 1–I, welches einerseits einen Impuls an den Drehwähler Rel DW weitergibt, anderseits einen Widerstand 200 Ω/2 Watt dem Kondensator C_L parallel schaltet. Dadurch wird die Entladung dieses

Literatur S. 147.

Kondensators wesentlich beschleunigt. Die einmalige Einstellung des Zündeinsatzpunktes und des Zündstromes erfolgt über die Potentiometer P_1–P_3, die Messung der entsprechenden Spannungen und Ströme erfolgt dabei über die Telefonklinken K_1–K_3. Bei Röhrenwechsel sind diese Abgleichungen natürlich neu vorzunehmen um Übereinstimmung mit den Geräteskalen zu erreichen. Die Einstellung der gewünschten Sammelzeiten erfolgt mit den Potentiometer P_4 (4 Sek. bis 12 Min.) und P_5 (10 Min. bis 20 Min.). P_8 wird auf genau 15 Min. eingestellt und dient zur Zeitvervielfachung (7 mal 15 Min. plus der an P_4 bzw. P_5 eingestellten Zeit). P_6 erlaubt die Regelung der Ablauf- und Abtropfzeit zwischen 4 und 13 Sek. Diese Zwischenzeiten wurden vorgesehen, um einerseits verschieden zähen Flüssigkeiten ein vollständiges Auslaufen aus dem Messgefäss zu ermöglichen, anderseits bei bereits geschlossenem Auslaufventil ein völliges Abtropfen der letzten Flüssigkeitsreste aus dem Zuteilerrohr sicherzustellen. Die genannten Potentiometer werden entsprechend der Funktionsfolge durch die Drehwählerbahn DWB 1 eingeschaltet, steuern die Gittervorspannung der EF80 und beeinflussen dadurch den Ladestrom von C_L. Der Schalter S_2 erlaubt die Abschaltung der beiden stetig veränderlichen Sammelzeiten, während die Vervielfachung der 15-Min.-Zeit durch einen doppelpoligen Mehrfingerschalter S_6 stufenweise zwischen den Werten 0 und 7 verändert werden kann. Der Schalter S_3 dient zur Umschaltung auf kurze (4 Sek. bis 12 Min.) oder lange (10 Min. bis 20 Min.) Sammelzeiten. Befindet sich der Schaltarm des Drehwählers in der gezeichneten Stellung, so wird C_L allmählich aufgeladen bis zur Erreichung des Zündpunktes. Der einsetzende Zündstrom bringt Rel 1-I zum Anziehen, der sich dadurch schliessende Kontakt gibt an den Drehwähler Rel DW einen Impuls weiter und der Schaltarm springt in die nächste Stellung. Auf der Bahn DWB 1 wird dadurch die fest eingestellte 15 Min.-Zeit eingeschaltet, deren Wiederholungszahl durch die Stellung von S_6 bestimmt wird. Die oberen Schaltfinger legen dabei jeweils das Sicherheitsventil SiV an Spannung, wodurch dieses geöffnet wird. Die unteren Schaltfinger legen hingegenüber die nicht benötigten Kontakte der Drehwählerbahn DWB 2 und über einen Ruhekontakt an Rel DW das Relais Rel 1–II an Spannung. Dessen Kontaktsätze sind dem Relais Rel 1–I parallel geschaltet, so dass beim Ansprechen jeweils ein Impuls an Rel DW weitergegeben wird. Auf diese Weise werden die nicht benötigten Schaltstellungen des Drehwählers übersprungen. Erreicht der Schaltarm an DWB 1 den Kontakt 9, so wird die Auslaufzeit, an Kontakt 10 die gleiche Zeit als Abtropfzeit eingeschaltet. Durch die korrespondierende Bahn DWB 2 wird gleichzeitig das Sicherheitsventil SiV abgeschaltet und auf Stellung 9 das Auslaufventil SaV an Spannung gelegt, wodurch dieses geöffnet wird. Beim Weiterschalten auf Kontakt 10 schliesst es sich wieder. Wird SaV von Hand aus offengehalten oder überhaupt nicht verwendet, so können durch Schliessen von S_4 beide Drehwählerstellungen übersprungen werden, wie dies für die Zeitvervielfältigung schon beschrieben wurde. In der Stellung 11 der Schaltarme wird an DWB 1 die Anodenspannung der EF80 unterbrochen, diese Röhre also gesperrt. An DWB 2 wird über das Relais Rel 3 der Motor Mot. eingeschaltet, der den Teller mit den Auffanggefässen weiterdreht. Am Umfang dieses Tellers sind Nocken eingefeilt, die die Kontaktsätze T_1 und T_2 betätigen (Vgl. Fig. 5, Mitte links). T_1 ist für den Motor ein Selbsthaltekontakt, der einem Kontakt von Rel 3 parallel liegt. T_2 liegt mit dem zweiten Kontaktpaar von Rel 3 in Serie, spricht erst kurz nach T_1 an und gibt über Rel I–II and den Drehwähler einen Fortschaltimpuls. Damit wird über DWB 1 wieder auf Sammeln eingestellt und an DWB 2 das Sicherheitsventil SiV geöffnet. Der Motor dreht den Teller solange, bis T_1 über die Nocke abgeglitten ist und dadurch geöffnet wird. Die Verbindung zwischen Motor und Teller wird durch eine doppelte Schneckenübersetzung und eine kleine Gummirolle hergestellt, die als Friktionskupplung unmittelbar am Tellerumfang angreift. Für sehr kurze Sammelzeiten ist diese Art des Transportes wegen des etwas langsamen Vorschubes allerdings nicht ganz geeignet und eine Ausführung ähnlich der von GRUBHOFER UND LWOWSKI wäre vorzuziehen. Ein Sprungmechanismus, wie er bei manchen Industriegeräten verwendet wird, ist wegen des ruckartigen Tranpsortes unzweckmässig. Die Transporteinrichtung unseres Gerätes ist in Fig. 1 und 5 gut ersichtlich.

Wie aus dem Gesamtschaltbild Fig. 6 hervorgeht, sind einige Relais durch Elektrolytkondensatoren überbrückt, um auch bei kurzen Impulsen ein längerdauerndes Anziehen zu gewährleisten. Die entsprechenden Zeitkonstanten sollten bei einigen Zehntelsekunden liegen. Hochbelastete Kontakte werden zur Funkenlöschung ebenfalls kapazitiv überbrückt. Die dritte Bahn des Drehwählers (DWB 3) betätigt Signallämpchen, die die jeweilige Wählerstellung anzeigen und mit S_5 abgeschaltet werden können. Der mit $S_{I\text{-}I\,u\,II}$ bezeichnete Telefonkippschalter stellt in der gezeichneten Stellung die Verbindung zwischen dem Netzteil und dem übrigen Gerät her, in Mittelstellung ist diese Verbindung getrennt und die unterste Stellung (Taster!) ermöglicht das Weiterschalten des Drehwählers auf einen gewünschten Kontakt, ohne dass das gesamte Gerät in Funktion tritt.

Aus Fig. 2 geht der mechanische Aufbau des Steuergerätes hervor. Die einzelnen Bauelemente wurden auf zwei Chassisblechen (Alu, 1 mm stark) und der Vorderwand untergebracht. Die Verbindung zwischen diesen drei Hauptbestandteilen erfolgt durch Vielfachstecker. Durch dieses "Baukastenprinzip" sind alle Teile leicht zugänglich und können trotzdem auf kleinsten Raum untergebracht werden. Die Fig. 2 zeigt von links nach rechts:

Oben: Netztrafo, Gleichrichterrohr, Gastriode, hinter beiden den Sieb-elko, rechts die EF80 und dahinter den isoliert aufgesetzten Ladekondensator (auf gute Isolation zwischen ihm und dem Chassis ist besonders zu achten!)

Mitte: Unter dem Trafo die Potentiometer P_2 und P_3. Am unteren Chassis befindet sich rechts oben P_1 und die Klinke K_1.

Unten: links das Netzrelais Rel N und rechts das Drehwählrelais.

(Alle Abbildungen zeigen eine ältere Ausführung des Fraktionssammlers, der als längste Sammelzeit 28 Min. hatte. Es fehlt daher der Schalter S_6).

Üblicherweise werden zur Zeitschaltung Entladungsvorgänge verwendet, da sich diese auch bei langen Zeiten leichter beherrschen lassen. Wird jedoch, wie hier, eine rasche Aufeinanderfolge verschiedener Schaltzeiten verlangt, so versagt diese Methode wegen der erforderlichen Aufladezeiten. Wir haben uns deshalb für einen Aufladevorgang entschlossen, obwohl dieser nur bis zu etwa 30 Min. einigermassen

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reproduzierbar ist. Eine wesentlich längere Schaltzeit bei tragbarer Toleranz ist auch mit einfachem Stabilisierungsmitteln nicht zu erreichen. Bei langen Zeiten muss also die Zeitvervielfachung mit S_6 in Kauf genommen werden.

B. Die Mess- und Sammeleinrichtung

Fig. 3 und Fig. 4 zeigen diesen Teil recht übersichtlich. Links oben sieht man das untere Säulenende und das daran anschliessende Sicherheitsventil. Dieses mündet in ein Trichterchen, welches seitlich an ein weiteres U-förmiges Rohr (Bildmitte) angesetzt ist. Im linken Schenkel dieses U-Rohres befindet sich das Auslauf- oder Sammelventil SaV, darüber der Betätigungsmagnet (umgebautes 24 V-Tauchkernrelais), darunter der Auslauf zum Zuteilröhrchen. Der rechte Schenkel nimmt einen gläsernen Schwimmer auf. Die beiden in Fig. 6 gezeichneten Platinspitzen tauchen in einige Tropfen verdünnte Schwefelsäure (Quecksilber neigt zur Häutchenbildung und ist deshalb nicht geeignet). Durch Heben und Senken dieses Schwimmers samt den beiden Kontakten mittels einer Schraubenspindel (in Fig. 3 neben dem Magnet) lässt sich das zu sammelnde Volumen leicht und stufenlos einstellen. Bei Eintauchen der beiden Pt-Spitzen in die Schwefelsäure wird der Kontakt Sch (Fig. 6) geschlossen und damit über Rel I–II an Rel DW ein Fortschaltimpuls gegeben. Dieser Impuls wird nur in den Wählerstellungen 1 bis 8 wirksam, damit ist ein Fehlimpuls zu anderen Zeiten (etwa während des Auslaufens oder des Weiterdrehens) unmöglich. Da die Volumensteuerung dauernd wirksam bleibt, also auch wenn mit Sammeln nach Zeit gearbeitet wird, bedeutet ein Ausfall der Zeitsteuerung lediglich eine automatische Umschaltung auf Volumensammeln und gleichzeitig damit eine Sicherung gegen ein Überlaufen des Sammelgefässes.

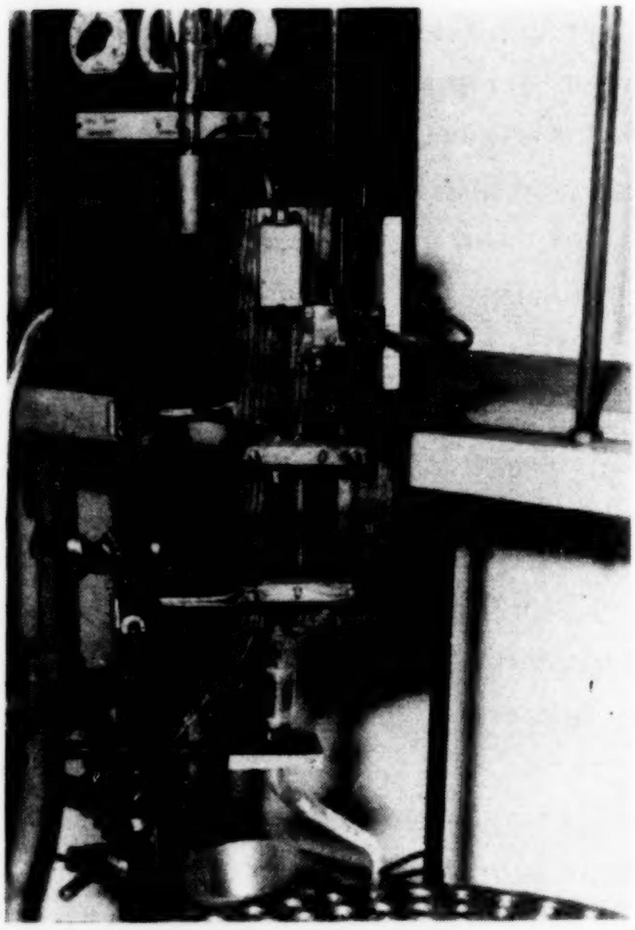

Fig. 3. Säulenende mit Sicherheitsventil, Mess- und Sammelteil, Volumensteierung und Zuteilröhrchen.

An Stelle des Schwimmers kann natürlich jede andere Dosierungseinrichtung treten, wenn sie nur bei Erreichen des eingestellten Wertes einen Impuls an Rel I-II abgeben kann. Es kann dies z.B. ein Tropfenzähler, eine Photozelle mit Verstärker oder ein integrierender Geigerzähler u.a.m. sein. Wir glauben, mit diesen Möglichkeiten die Anwendungsbreite des Gerätes wesentlich erweitert zu haben.

C. Die Auffang- und Transporteinrichtung

Die aus dem Sammelgefäss auslaufende Flüssigkeit gelangt zunächst in ein Trichterchen mit gebogenem Rohr, welches um eine senkrechte Achse leicht drehbar an-

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geordnet ist (siehe Fig. 5, Mitte oben). Die Mündung des Zuteilerröhrchens steht jeweils über einem Vorlagegefäss (Eprouvette o.ä.) und wird durch Umlenkstege nach dem Füllen eines Kreises Eprouvetten selbsttätig auf die nächsten Kreise geschwenkt. Die in Fig. 5 sichtbaren Stege der ersten Geräteausführung haben von

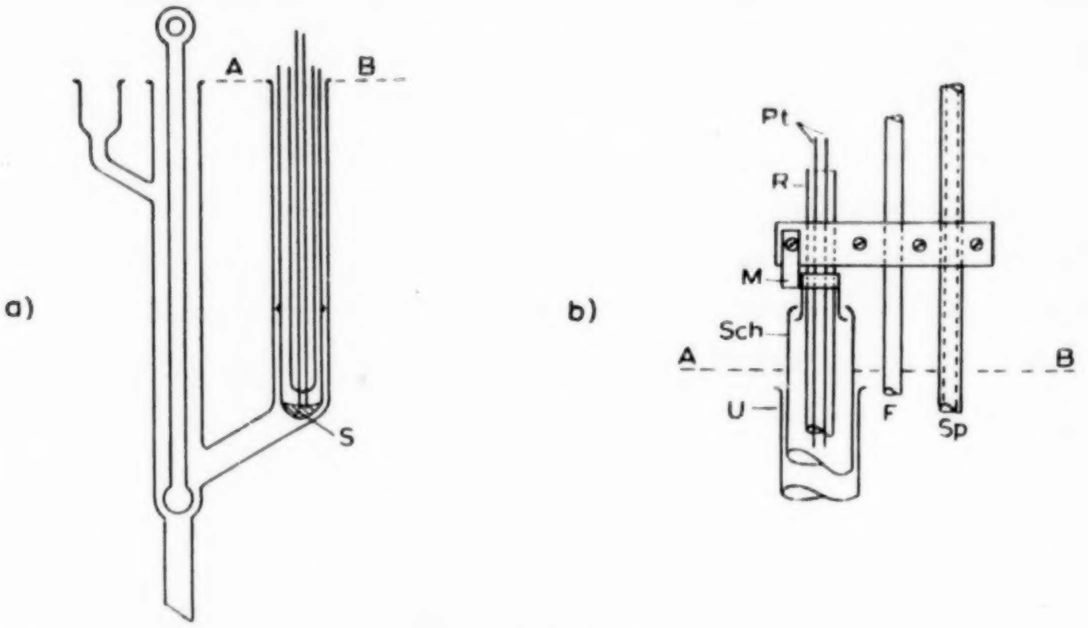

Fig. 4a und b. Mess- und Sammelteil. (a) Gesamtskizze; (b) Schwimmerbefestigung. S: Schwefelsäure; F: Führungsstab; U: Sammelgefäss; Pt: Platinkontakte; Sp: Schraubenspindel; Sch: Schwimmkörper; R: Einschmelzröhrchen; M: Messingfeder.
(Der Schwimmer Sch gleitet zwischen oberem und unterem Anschlag der Feder M, dadurch wird einerseits ein Herabfallen und anderseits ein zu hohes Aufschwimmen und Beschädigung der Platinkontakte verhindert. Diese sind an der Durchschmelzstelle samt dem Röhrchenende schwach paraffiniert um ein Haften der Schwefelsäure zu unterbinden).

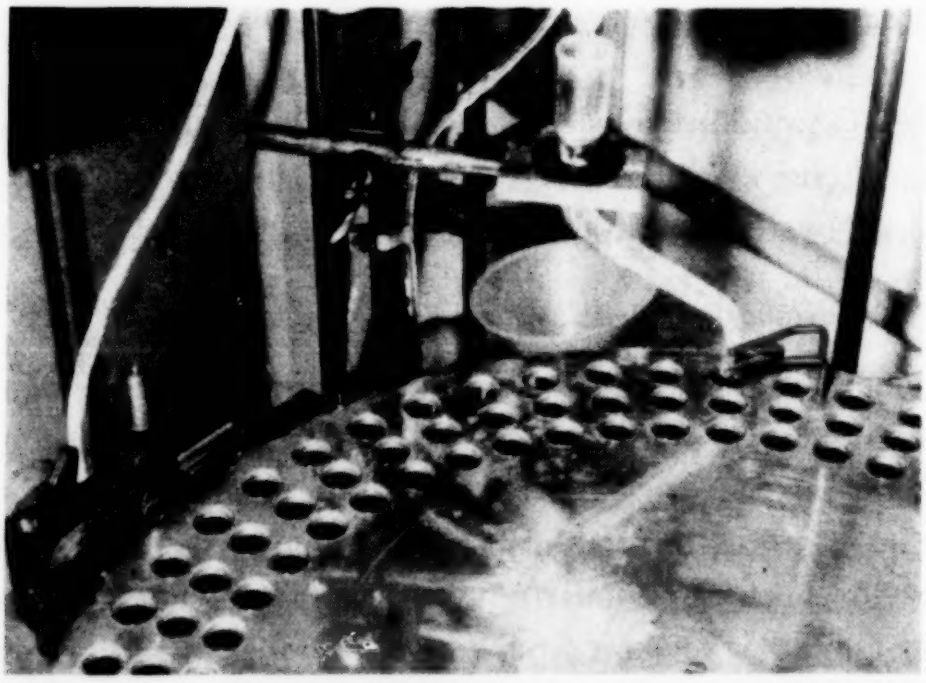

Fig. 5. Zuteilröhrchen. Teller mit Auffanggefässen, Umlenkstegen und Randnocken sowie Kontaktfedersatz und Motor.

aussen nach innen gelenkt, während sie beim neuen Fraktionssammler vom inneren Kreis auf den äusseren und nach dessen Füllung über einen Trichter leiten. Auf diese Weise kann nach beendeter Füllung aller Proberöhrchen (150–200) das weiter ablaufende Eluat in einem geeigneten Gefäss aufgefangen werden. Es ist jedoch auch

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möglich, beim Ausschwenken über den Tellerrand mittels eines Kontaktes den Fraktionssammler ausser Betrieb zu setzen.

Fig. 5 zeigt deutlich die Schaltnocken am Tellerumfang, sowie den damit bedienten Kontaktsatz, der am Fühlhebel eine kleine Rolle zur Verminderung der Reibung beim Vorbeigleiten der Nocken trägt. In der linken unteren Ecke ist noch der Motor mit der Gummiantriebsrolle sichtbar. Die Funktion dieser Teile wurde bereits früher eingehend erörtert. Erwähnt mag noch werden, dass der Teller, aus stärkerem Alu-Blech bestehend, kugelgelagert ist.

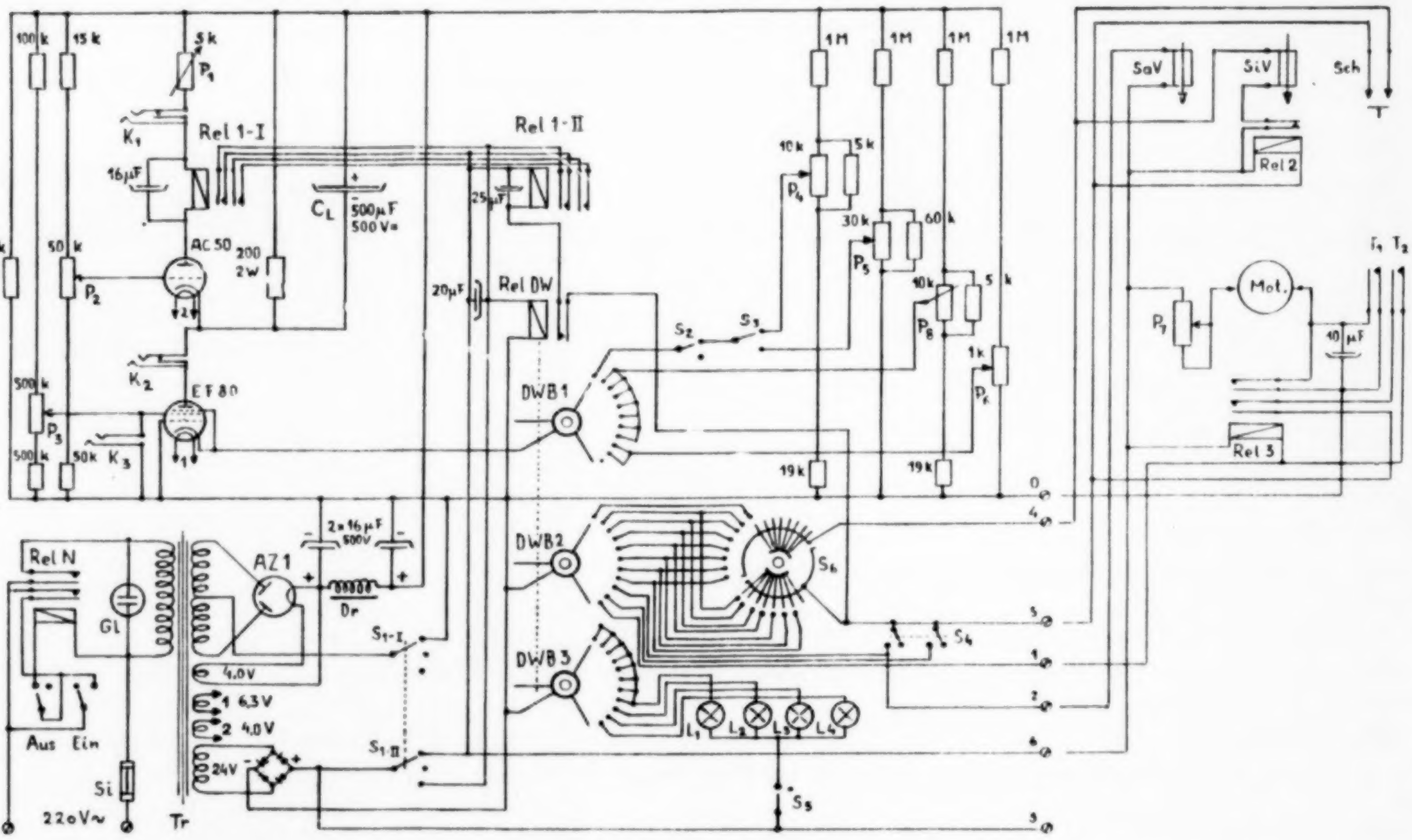
100 k
15 k
5 k
P1
K1
Rel 1-I
16µF
C_L
500µF 500V=
Rel 1-II
25µF
1M
1M
1M
1M
10k
5k
P4
30k
60k
P5
10k
5k
P6
1k
SaV
SiV
Sch
Rel 2
T1 T2
50k 4W
50 k
P2
AC 50
200 2W
20µF
Rel DW
S2
S3
Mot.
P7
10µF
500 k
P3
500 k
50k
K2
K3
EF 80
DWB 1
19k
19k
Rel 3
0
4
Rel N
Gl
AZ 1
2×16µF 500V
Dr
DWB2
S6
3
S1-I
S4
1
DWB 3
4,0V
1 6,3V
2 4,0V
24V
2
S1-II
L1
L2
L3
L4
6
S5
Aus Ein
Si
220V~
Tr
5

Fig. 6. Gesamtschaltbild des Fraktionssammlers (Zeichenerklärung siehe Text).

D. Die Säule mit dem Sicherheitsventil

Das hier beschriebene Gerät erlaubt selbstverständlich die Verwendung jeder beliebigen Säule, sowohl bezüglich der Grösse als auch der Durchlaufgeschwindigkeit. Wichtig ist lediglich das Sicherheitsventil SiV (Fig. 3 und 6). Es handelt sich um ein Kugelventil in Ganzglasausführung. Im Ventilschaft ist ein Weicheisenkern eingeschmolzen, der durch ein Solenoid (umgebautes 24 V-Tauchkernrelais) das Ventil öffnen kann. Bei Stromlosigkeit der Spule sperrt dieses Ventil daher den Auslauf aus der Säule. Das Solenoid wird nun *nur* während der Sammelperiode erregt, zu allen anderen Zeiten ist es geschlossen, es kann daher auch nur zu dieser Zeit Flüssigkeit aus der Säule in das Messgefäss gelangen. Tritt während des Sammelns eine Störung in der Zeitsteuerung ein, so wird, wie früher dargelegt, die Volumensteuerung wirksam, die den Drehwähler in die Stellung "Auslaufen" springen lässt. Dort aber ist das Sicherheitsventil bereits geschlossen und bleibt es, bis der Wähler wieder auf "Sammeln" kommt. Während dieser Zwischenstellungen kann also aus der Säule keine

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Flüssigkeit nachgeliefert werden. Durch die gegenseitige Verriegelung der einzelnen wichtigen Kontakte ist aber auch eine unbeabsichtigte Auslösung irgend eines Schaltvorganges zur falschen Zeit sehr wirksam unterbunden.

Es mag vielleicht der Einwand erhoben werden, dass eine Unterbrechung des Flüssigkeitsstromes in der Säule eine Verwaschung der Zonen durch Diffusion herbeiführen kann. Dem lässt sich entgegehalten, dass diese Unterbrechung normalerweise nur ganz kurzzeitig ist. Bei einem Totalausfall des Fraktionssammlers, etwa durch Netzausfall, halten wir es jedoch für wesentlich zweckmässiger, die oft sehr wertvolle Substanz in der Säule zu bewahren, woraus sie immerhin wieder gewonnen werden kann, als sie ohne verwaschene Zonen über den Fussboden verteilt zu finden.

Es kann abschliessend jedenfalls gesagt werden, dass sich das hier beschriebene Gerät leicht und billig nachbauen lässt, gut reproduzierbare Ergebnisse liefert und sich bei uns in oft wochenlangem Dauerbetrieb bestens bewährt hat. Fig. 1 bringt eine Anordnung, wie sie für verschiedene Zwecke bei uns seit längerer Zeit benützt wird.

DANK

Unserem Institutsmechaniker, Herrn G. GAAB, mächten wir an dieser Stelle für seine Mühe bei der Herstellung des Gerätes danken.

ZUSAMMENFASSUNG

Es wird ein Fraktionssammler beschrieben, der aus handelsüblichen Teilen aufgebaut ist, wobei auf grösstmögliche Betriebssicherheit geachtet wurde. Das Sammeln kann sowohl nach Zeit als auch nach Volumen erfolgen. Sie Sammelzeit ist zwischen etwa 4 Sekunden und 2 Stunden stufenlos regelbar. Der korrosionsempfindliche Steuerteil ist durch Kabel mit der Säule und der Auffangvorrichtung verbunden und kann daher in beliebiger Entfernung von den letzteren untergebracht werden.

SUMMARY

A description is given of a fraction collector, which is constructed from parts that can be obtained commercially. The construction is such that the apparatus is extremely reliable. Fractions can be collected at definite time intervals or when definite volumes have accumulated. The collection time can be regulated continuously, for periods ranging from about 4 seconds to 2 hours. The regulator, which is susceptible to corrosion, is connected to the column and the collector by means of a cable, so that it can be placed at any desired distance from these parts.

LITERATUR

[1] N. GRUBHOFER UND W. LWOWSKI, *Chem.-Ing.-Tech.*, 28 (1956) 579. Dort auch weitere Literatur.

Eingegangen den 29. Oktober 1958

A FLAME IONIZATION DETECTOR FOR GAS CHROMATOGRAPHY

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This paper describes a modified form of the single jet flame ionization detector of McWilliam and Dewar[1]. The difference between the modified and the original form of the apparatus lies mainly in the electrical circuit of the amplifier. With the amplifying circuits described below the modified form of the detector has exhibited good base line stability and high sensitivity.

The realizable sensitivity of a detector for gas chromatography depends upon the acceptable signal to noise ratio as presented on the recorder chart, therefore the base line drift and noise level of detecting and measuring apparatus should be as low as possible. It is convenient to consider separately the two main parts of a flame ionization detector, the combustion chamber (detector) and electronic amplifier.

A reliable detector is simple to design and construct but a suitable amplifier is more of a problem, as it requires to have a very high input resistance, low output resistance, low noise level, and a high degree of linearity between input and output if peak areas are to be measured.

The amplifiers to be described have all these features, several have been constructed, the measured noise level of one is stated, and a gas chromatogram using this with a flame detector is shown.

THE DETECTOR

Details of the construction of the detector are shown in the scale drawing (Fig. 1) which is self-explanatory.

Normally hydrogen has been used as the carrier gas, but the apparatus is so constructed that nitrogen may be employed when necessary and mixed with hydrogen after its emergence from the column. The narrow bore of the three way tube between the column and the jet ensures that when nitrogen is used there is no diffusion of hydrogen into the column and no escape of eluted material through the tube supplying the hydrogen. The air which is supplied to the combustion chamber is filtered through glass wool, because, as McWilliam and Dewar have observed, even small particles of dust cause large disturbances.

It has been found that electrical insulation of a high order between the probe (gauze) and earth, and the use of stationary phases of very low volatility are essential for good base line stability. The first of these requirements is met by using insulators with a long tracking path over their surface and made of ceramic, which is a better

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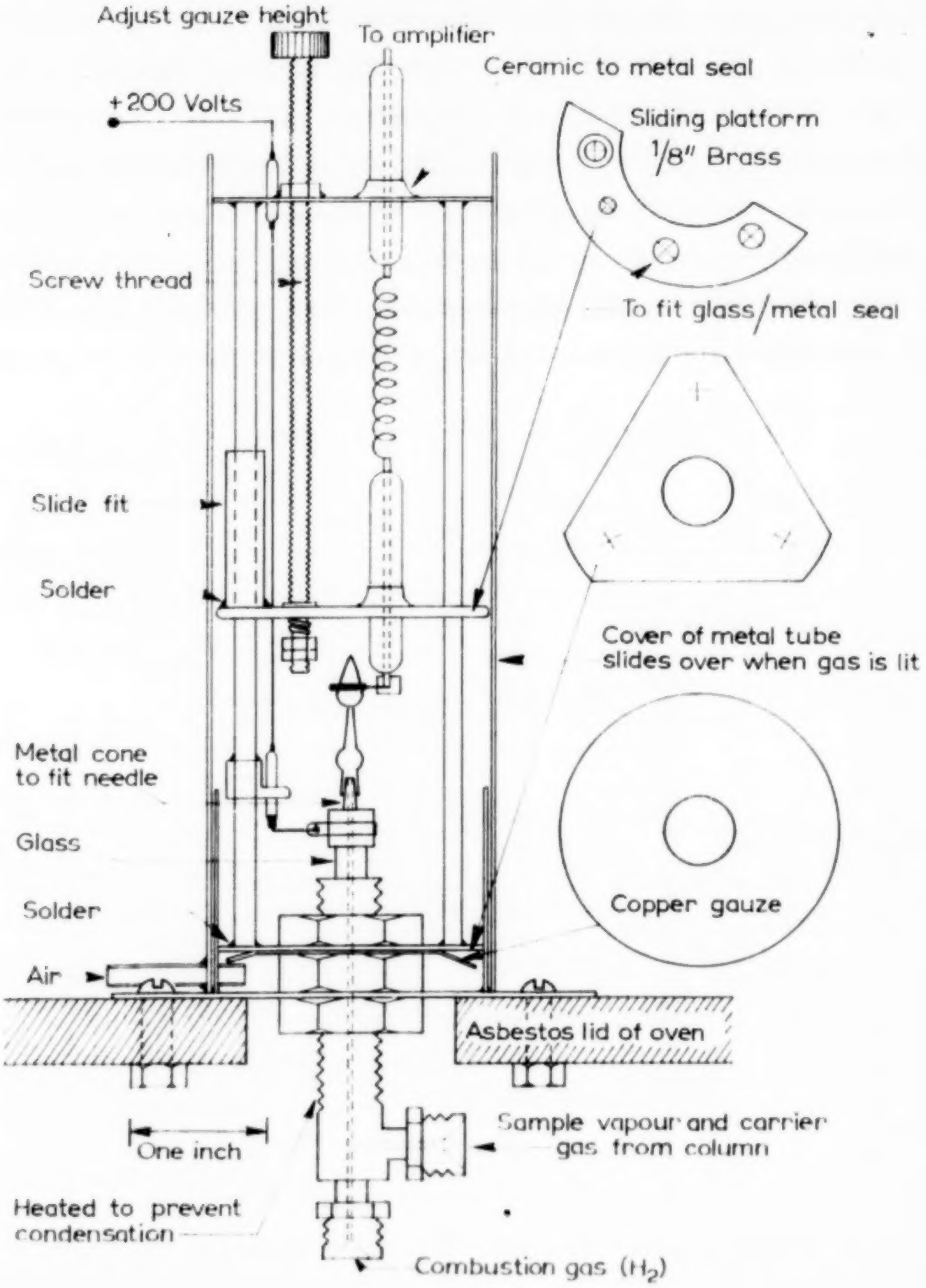
Adjust gauze height
To amplifier
Ceramic to metal seal
+200 Volts
Sliding platform
1/8" Brass
Screw thread
To fit glass/metal seal
Slide fit
Solder
Cover of metal tube
slides over when gas is lit
Metal cone
to fit needle
Glass
Solder
Copper gauze
Air
Asbestos lid of oven
One inch
Sample vapour and carrier
gas from column
Heated to prevent
condensation
Combustion gas (H_2)

Fig. 1.

insulator than glass at high temperatures. Ocasionally it was noticed that the insulation deteriorated owing to the deposition of products of combustion on the ceramic-to-metal seals. To obviate this a metal screen (not shown in the diagram) was inserted between the flame and the seals.

AMPLIFIERS

It is necessary when designing an amplifier for this application to consider the requirements of the pen recorder which is to be used. Most laboratories using gas chromatography apparatus have potentiometric pen recorders intended for use with thermal conductivity cells. Such recorders have a sensitivity of one or two mV full scale deflection and therefore have a limitation regarding the value of electrical resistance which may be connected across their input terminals. Usually 500 Ω is the maximum which may be employed if the correct pen response time is to be obtained. It is of

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course desirable to keep the electrical circuits simple in the interests of reliability, but unfortunately it is not possible to design an amplifier using only one battery-type electrometer valve if it is to have a voltage gain of unity and an output resistance of 500 Ω. Since it is desirable to have high linearity between input and output voltage for an input of + and — 25 V, it is more convenient to use one of the multi-valve circuits to be described.

Alternative amplifying circuits are shown in Figs. 2 and 3. The first is a modification of a circuit described by SCROGGIE[2]. It is often assumed that an amplifier of this type is inherently stable, but it must be remembered that it is a high voltage gain amplifier with negative feedback, and phase shift within the amplifier may possibly cause oscillation of a high amplitude. If this happens it may well be that the amplifier is operating outside its correct characteristics, saturated, with V_1 control grid current flowing during part of each cycle of oscillation, exhibiting blocking and low input resistance. 50 c/sec will be introduced into the circuit if the 6.3 heater voltage is not at a definite fixed potential, and it should be connected to a valve cathode (V_1) as shown. As a precaution against A.C. pickup on the input circuits, and amplifier instability, the capacitor C_2 will short out alternating voltage, whatever its source. The zero stability of this type of amplifier also requires that the heaters of V_1, V_2 and V_3 be "aged" by running them for several hundred hours.

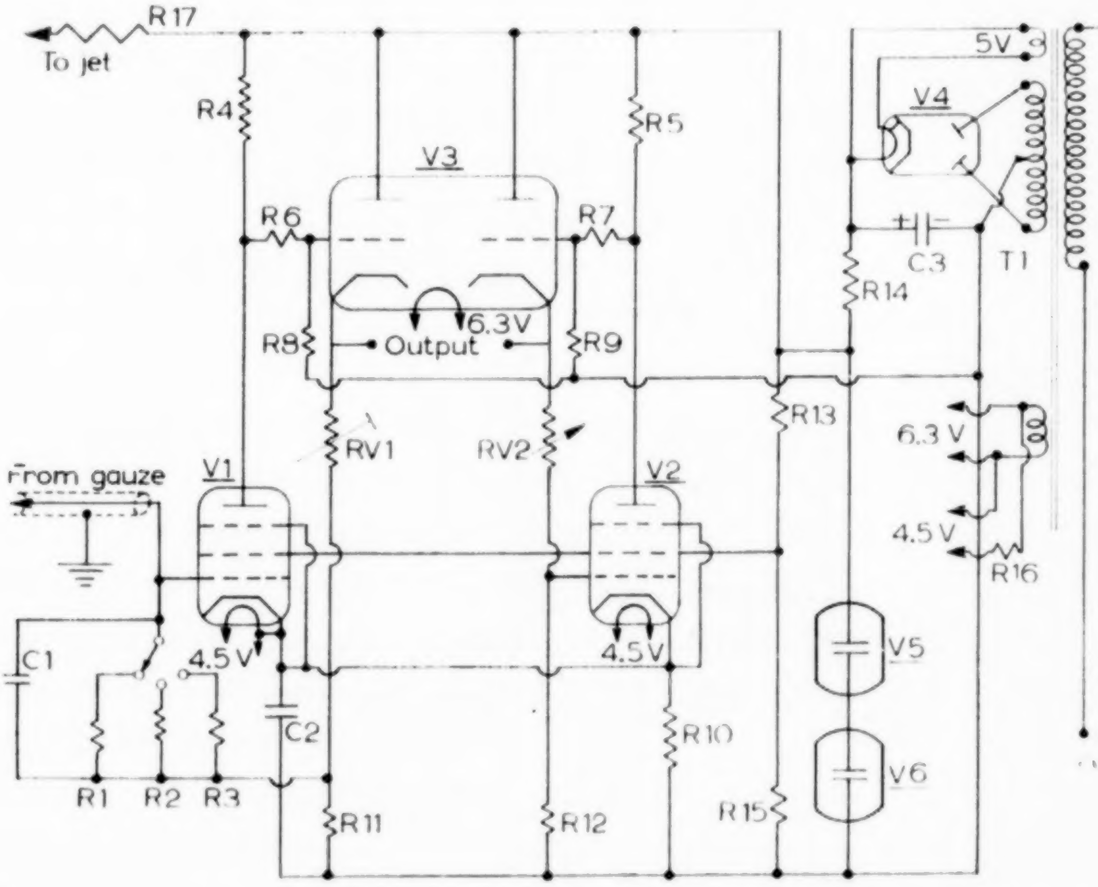

Fig. 2. Resistors: 1. $1 \cdot 10^9\,\Omega$; 2. $3 \cdot 10^9\,\Omega$; 3. $1 \cdot 10^{10}\,\Omega$; 4, 5. 820 kΩ, 2%, ½ W; 6, 7. 680 kΩ, 2%, ½ W; 8, 9. 2.2 MΩ, 5%, ½ W; 10. 470 kΩ, 5%, ½ W; 11, 12. 12 kΩ, 5%, 2 W; 13, 15. 82 kΩ, 5% 1 W; 14. 2.2 kΩ, 10%, 5 W; 16. 5.6 Ω, 10%, 2 W; 17. 1 MΩ, 20%, ½ W. RV_1: 500 Ω. Preset gain control. RV_2: 500 Ω. Variable zero control (panel mounting). Capacitors: C_1. Polystyrene, value as required (0.001 μF?); C_2. 0.1 μF, 150 V; C_3. 8 μF, 500 V. T_1 Mains transformer. Primary as required for local mains supply. Secondary: 350–350 V, 80 mA; 6.3 V, 2 A; 5.0 V, 2 A. Valves: V_1 and V_2. Type ME1400 (Mullard); V_3. Type ECC81 (Mullard) or 12AT7; V_4. Type 5Z4; V_5. Type VR/150/30; V_6. Type VR105/30. Input switch: One pole 3 way high insulation.

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A potentiometric pen recorder is connected to the output terminals of the amplifier through a suitable voltage attenuator. A 1 mA moving coil recorder can be used equally well with this type of amplifier.

Since the recorders which were available for use with the amplifier would not tolerate more than a few per cent of their input signal to consist of alternating voltage of supply frequency, A.C. pickup on the input of the amplifier must be filtered out. The amplifier was connected to the collector probe by a few metres of 75 Ω coaxial cable of approximately 100 $\mu\mu$F capacity per metre, which together with the input resistor used had a time constant of 1 or 2 seconds, sufficient to attenuate the A.C. pickup without appreciably increasing the response time of the pen recorder. Further capacitance can be made available, if necessary, by the use of polystyrene capacitors connected in parallel with the high value input resistors. These capacitors are shown as C_1 in the amplifier circuits.

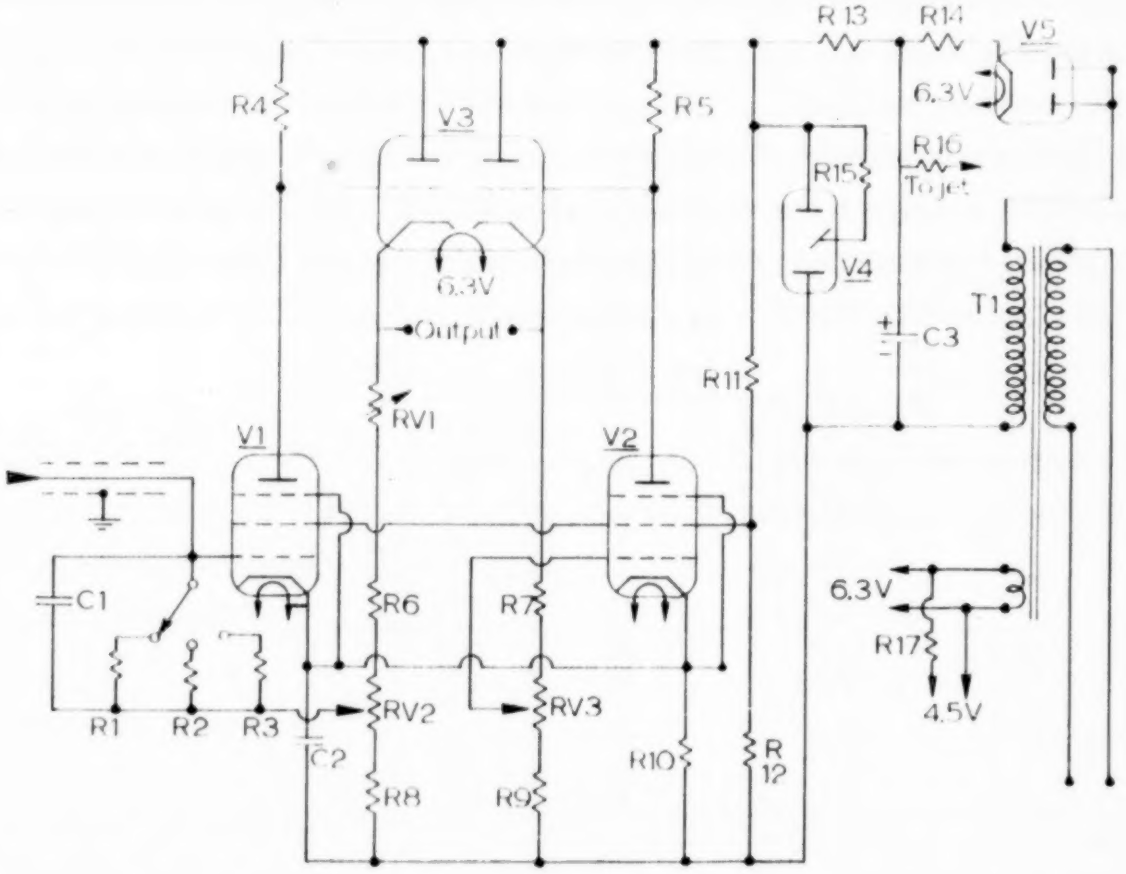

Fig. 3. Resistors: 1. $1 \cdot 10^9\,\Omega$; 2. $3 \cdot 10^9\,\Omega$; 3. $1 \cdot 10^{10}\,\Omega$; 4, 5. 470 kΩ, ½ W, 2%; 6, 7. 33 kΩ, 5%, 1 W; 8, 9. 10 kΩ, 5%, 1 W; 10. 100 kΩ, 5%, ½ W; 11. 150 kΩ, 5%, ½ W; 12. 750 kΩ, 5%, ½ W; 13. 10 kΩ, 20%, 2 W; 14. 220 Ω, 20%, ½ W; 15, 16. 1 MΩ, 20%, ½ W; 17. 5.6 Ω, 10%, 2 W. RV_1: 2 kΩ. Variable zero control (panel mounting). RV_2: 1 kΩ. Preset zero control. RV_3: 1 kΩ Preset gain control. Capacitors: C_1. Polystyrene, value as required; C_2. 0.1 μF, 150 V; C_3. 16 μF. 500 V. T_1 Mains transformer. Primary as required. Secondary: 250 V, 30 mA; 6.3 V, 2 A. Valves: V_1 and V_2. ME1400 (Mullard); V_3. ECC81 (Mullard); V_4. 150B3 (Mullard); V_5 EZ80 (Mullard) or 6X4 or 6X5.

Each amplifier is housed in a metal box of about 8 l capacity, large enough to keep the stray magnetic field of the mains transformer away from the valves, which may be separately screened. If the transformer is rated at much higher output currents than the circuit requires, so that its core operates well away from magnetic saturation, and is supplied in a metal shroud or box, it will not cause trouble. The coaxial cable from the detector is taken to the amplifier through its front panel without plug or socket and direct to the control grid of the pentode valve V_1. The moving contact of a good quality ceramic switch is also taken directly to the grid. It is important that these input components should be kept clean and dry. The resistors are held with thermal shunts during soldering, their body being untouched, and they are mounted away from the other components in the interests of temperature stability. If a slow drift of the base line is observed it may be due to the fact that insufficient time has been allowed for all components in the amplifier to reach their final temperature, and half an hour or so should be allowed for this. Other possible causes are the use of low grade or overrun components, and the mounting of temperature sensitive resistors, such as the pentode anode load resistors R_4 and R_5 too close to warm components. If identical resistors are mounted close together the effects of their resistance change due to heating tend to be cancelled out by the circuit. The pentodes should be mounted close together and away from the other valves. All valve holders should be of good quality as leakage across pins could produce noise on the base line.

Base line instability due to circulating earth currents may be avoided by earthing

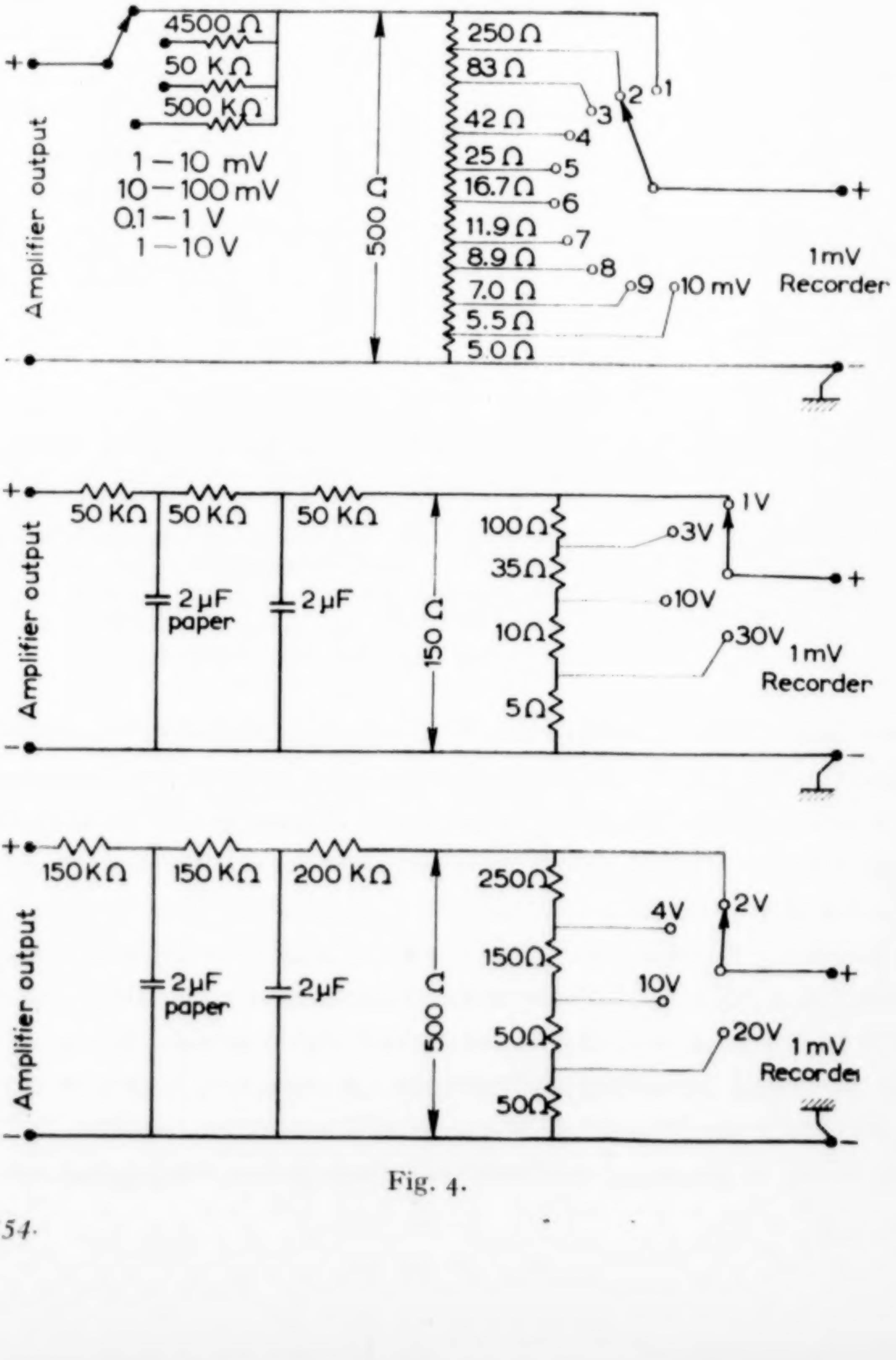
+
4500 Ω
50 KΩ
500 KΩ
1 — 10 mV
10 — 100 mV
0.1 — 1 V
1 — 10 V
Amplifier output
500 Ω
250 Ω
83 Ω
42 Ω
25 Ω
16.7 Ω
11.9 Ω
8.9 Ω
7.0 Ω
5.5 Ω
5.0 Ω
1
2
3
4
5
6
7
8
9
10 mV
+
1 mV
Recorder
+
50 KΩ
50 KΩ
50 KΩ
2 μF
paper
2 μF
150 Ω
100 Ω
35 Ω
10 Ω
5 Ω
1V
3V
10V
30V
+
1 mV
Recorder
Amplifier output
−
−
+
150 KΩ
150 KΩ
200 KΩ
2 μF
paper
2 μF
500 Ω
250 Ω
150 Ω
50 Ω
50 Ω
4V
2V
10V
20V
+
1 mV
Recorder
Amplifier output
−
−

Fig. 4.

the whole apparatus at one point, the negative input terminal of the pen recorder. The ceramic switch, high value resistors and pentode valves should be screened from dust, moisture, light and other radiation.

When the measurement of peak areas is required an Electo Methods integrator motor counter can be connected directly to the output terminals of the amplifier, which is particularly suitable for the purpose on account of its very low output resistance and linear input to output voltage over a wide range of input voltage. Since such a motor requires 6 or 12 V for full speed, depending on its type, an input resistor of 1000 to 3000 MΩ is required for normal sensitivity of detection. Although no resistor of more than 10,000 MΩ has been used for routine work, such a high value can be employed without adverse effect.

The second amplifying circuit with a voltage gain of four has a higher output resistance of about 500 Ω, and is suitable for use with a 12 or 24 V motor.

Both amplifiers supply a high voltage to the needle jet through a resistor of 1 MΩ.

VOLTAGE ATTENUATOR

Since several volts are produced at the output of either amplifier when an organic substance in the effluent stream burns, it is necessary to connect a voltage attenuator between the amplifier and the pen recorder.

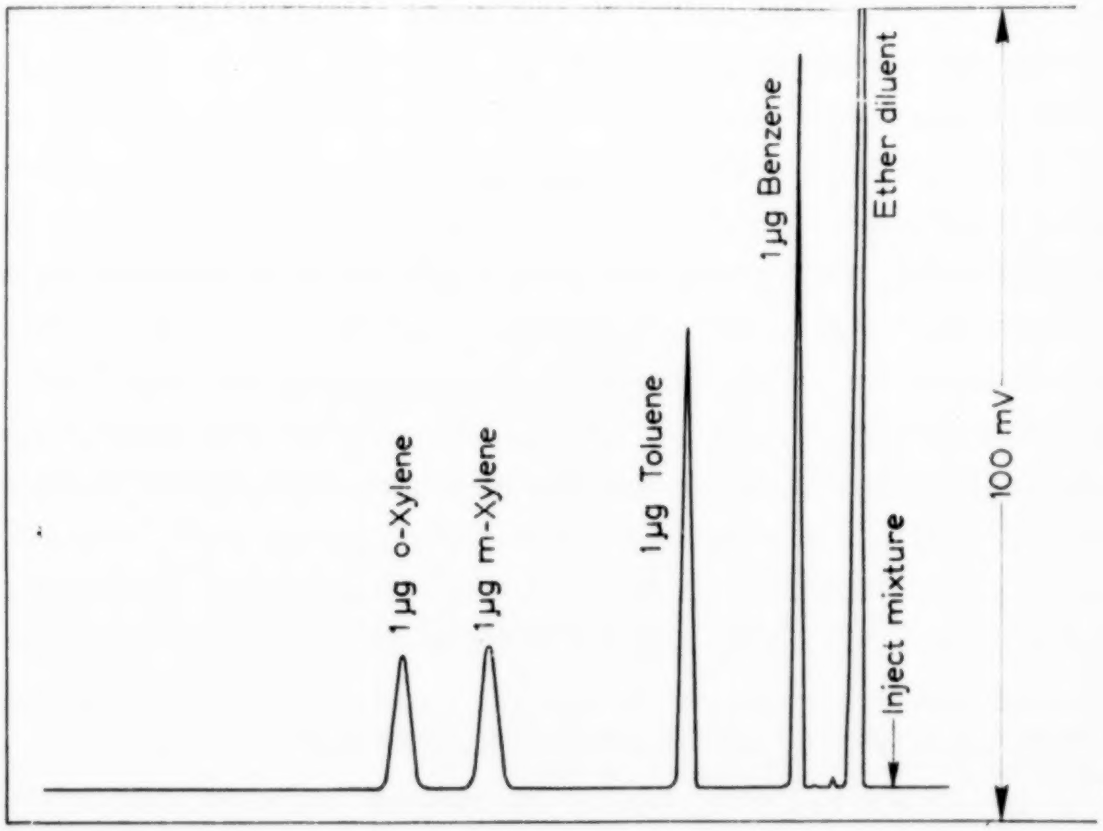

Fig. 5.

The attenuator circuits used with the amplifier are shown in Fig. 4. Small radio type switches are used. The odd and low value resistors are wound with insulated constantan wire. It is sometimes convenient to filter out A.C. by designing the attenuator to provide greater attenuation of A.C. than D.C., a feature of the second and third attenuator circuits.

An amplifier constructed to the circuit shown in Fig. 2 was tested for noise level and sensitivity to voltage fluctuations in the supply mains. It was found that changes of + and — 5% resulted in output fluctuations of + and — 2 mV. With a constant mains voltage the short term drift was within 200 μV, and the maximum deviation after 4 hours was 500 μV.

The amplifier was connected to the detector shown in Fig. 1 and the chromatogram shown in Fig. 5 was obtained with a mixture of approx. 1 μg each of benzene, toluene, and *m*- and *o*-xylene. The initial large peak is 1 mg of ether used as a diluent.

Using the sensitivity parameter of DIMBAT, PORTER AND STROSS[3], the measured sensitivity of the benzene peak of Fig. 5 is $S = 1.1 \cdot 10^6$ ml·mV/mg, and the signal to noise ratio 200 to 1.

The column used was a coiled copper tube 4 m long and 4.5 mm internal diameter, filled with a stationary phase of "Apiezon L" grease on firebrick, operated at a temperature of 100° and an inlet pressure of 1426 mm of Hg. The carrier gas was hydrogen, flowing at a rate of 31 ml/min. The chart paper speed was 1.97 min/cm and the area of the benzene peak 4.0 cm^2.

ACKNOWLEDGEMENTS

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SUMMARY

Multi-valve amplifying circuits for use with a gas chromatography flame ionization detector are described. With these amplifiers, designed to have a very high input and low output resistance and high degree of linearity between input and output, the detector exhibits high signal to noise ratio and sensitivity. The various possible causes of base line instability are discussed and the appropriate remedial measures indicated.

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ÉLECTROPHORÈSE SUR PAPIER DU PROTACTINIUM(V)

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La migration des ions sur une bande de papier imbibée d'électrolytes sous l'influence d'un gradient de potentiel, a été étudiée dès 1948 par WIELAND et ses collaborateurs[1], CREMER ET TISELIUS[2] et par DURRUM[3] pour la séparation d'acides aminés, de peptides et de protéines. Cette méthode a, par la suite, été généralisée et elle a permis la séparation de nombreuses substances ionisées. Certains livres passent en revue la littérature de l'électrophorèse; parmi ceux-ci, nous citerons plus particulièrement l'ouvrage de LEDERER[4].

En chimie minérale, de nombreuses séparations ont été effectuées soit avec des électrolytes faibles, mais complexants, tel que l'acide lactique[5], soit avec des électrolytes forts comme l'acide chlorhydrique ou l'acide bromhydrique[6,7]. En particulier, la séparation du niobium et du tantale a été réalisée par BRUNINX, EECKHOUT ET GILLIS[8]. La séparation et l'électromigration du protactinium n'ont pas été mentionnées jusqu'à présent.

De notre côté, nous avons étudié principalement le comportement électrophorétique du protactinium ainsi que celui des éléments suivants: Fe(III), U(VI), Pb(IV), Bi(III), Zr(IV), Ta(V), Nb(V), Ti(IV), Th(IV), Po(III). Nous avons utilisé l'acide fluorhydrique dilué comme électrolyte, ainsi que le mélange d'acides chlorhydrique et fluorhydrique.

CONSIDÉRATIONS THÉORIQUES

Une théorie de l'électromigration sur support poreux a été développée par CONSDEN, GORDON ET MARTIN[9]. Les facteurs particuliers qui influencent la migration dans les bandes de papier ont été étudiées par KUNKEL ET TISELIUS[10]. Il nous suffira de rappeler brièvement que le mouvement d'un ion placé sur une bande de papier dépend des facteurs suivants:

(a) de la charge des ions, qui elle-même est fonction de la structure du complexe, du pH et de la force ionique de la solution.

(b) du mouvement électro-osmotique, conséquence des charges portées par le papier. Celles-ci ont pour effet de produire un déplacement du liquide d'une électrode à l'autre. Les charges portées par le papier résultant principalement de la dissociation des groupements COOH de la cellulose, cet effet sera négligeable dans les milieux très acides, la dissociation du groupement acide organique étant, dans ces conditions, très faible.

(c) mouvement du liquide nécessaire pour compenser la perte d'électrolyte par évaporation, ainsi que celui dû à la saturation du papier au début de la manipulation: en conséquence, la vitesse du liquide est maximum au départ des réservoirs d'électrolytes et nulle au centre de la bande de papier.

MODE OPÉRATOIRE

Nous avons été obligés de modifier légèrement l'appareillage classique par suite de la présence d'acide fluorhydrique.

En présence d'acide fluorhydrique seul, électrolyte faible, nous avons utilisé un appareil dans lequel le papier est entouré d'air, dans une chambre en plexiglas saturée de la vapeur de l'électrolyte considéré (Fig. 1). Le dégagement de chaleur, avec un courant faible (quelques mA), est insuffisant pour gêner la séparation électrophorétique.

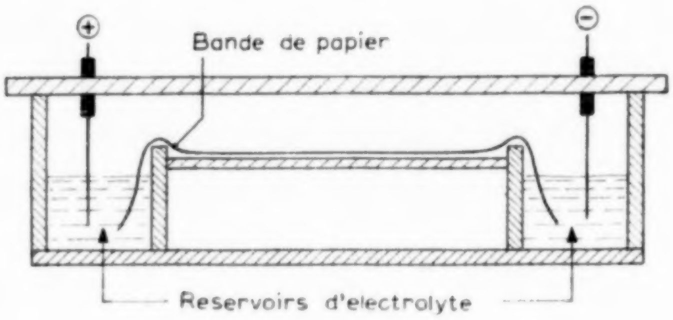

Fig. 1.

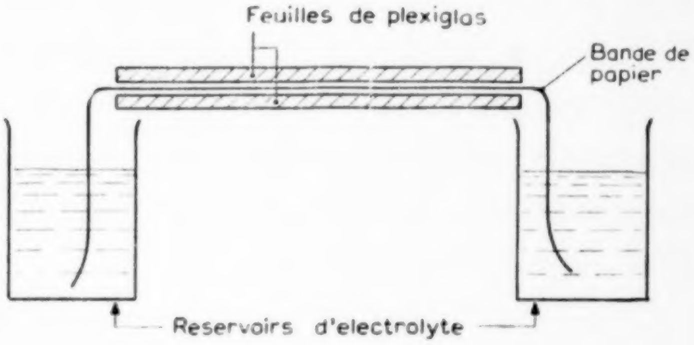

Fig. 2.

Lors de l'emploi d'électrolytes forts (mélanges HCl–HF), nous avons intercalé la feuille de papier entre deux plaques de plexiglas de 1 cm d'épaisseur. Ces plaques étaient maintenues par des pinces destinées à exercer une pression uniforme sur le papier. La Fig. 2 montre l'appareillage employé. Les deux réservoirs d'électrolytes sont constitués par des béchers en polyéthylènes.

Le papier employé était, dans tous les cas, du No. 302 Arches, celui-ci étant saturé, avant l'utilisation, par l'électrolyte.

PARTIE EXPÉRIMENTALE

1. *Electromigration en milieu HCl–HF*

Nous avons, tout d'abord, étudié l'électromigration des différents éléments envisagés précédemment avec un mélange HCl 0.6 *N*–HF 2 *N*. La présence d'HCl, acide fort,

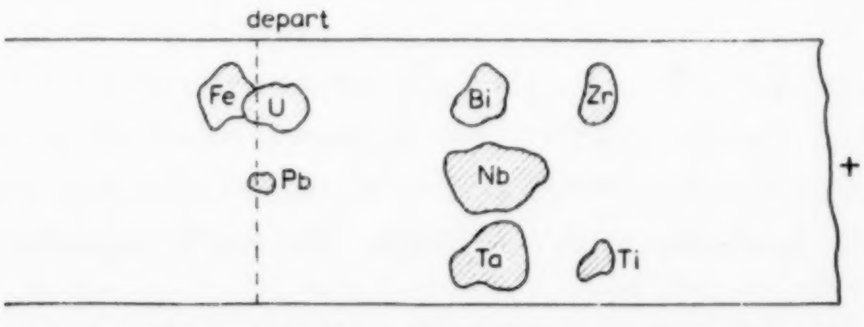

Fig. 3. HCl 0.6 *N*–HF 2*N*; voltage 150 V; durée 2 h.

diminue considérablement la résistivité du milieu. La tension aux bornes de la cuve était maintenue à 150 V et la durée de la manipulation égale à 2 h. La Fig. 3 et la Fig. 4 montrent le comportement de Fe(III), U(VI), Pb(IV), Bi(III), Nb(V), Ta(V), Ti(IV), Zr(IV), Th(IV), Pa(V). Dans ces conditions on constate que le fer est cationique; le plomb reste au point de départ par suite de l'insolubilité de son fluorure; tous les autres éléments, au contraire, ont un comportement nettement anionique. La Fig. 5 représente la migration du polonium dans les mêmes conditions. Le Tableau I résume l'ensemble de cette étude.

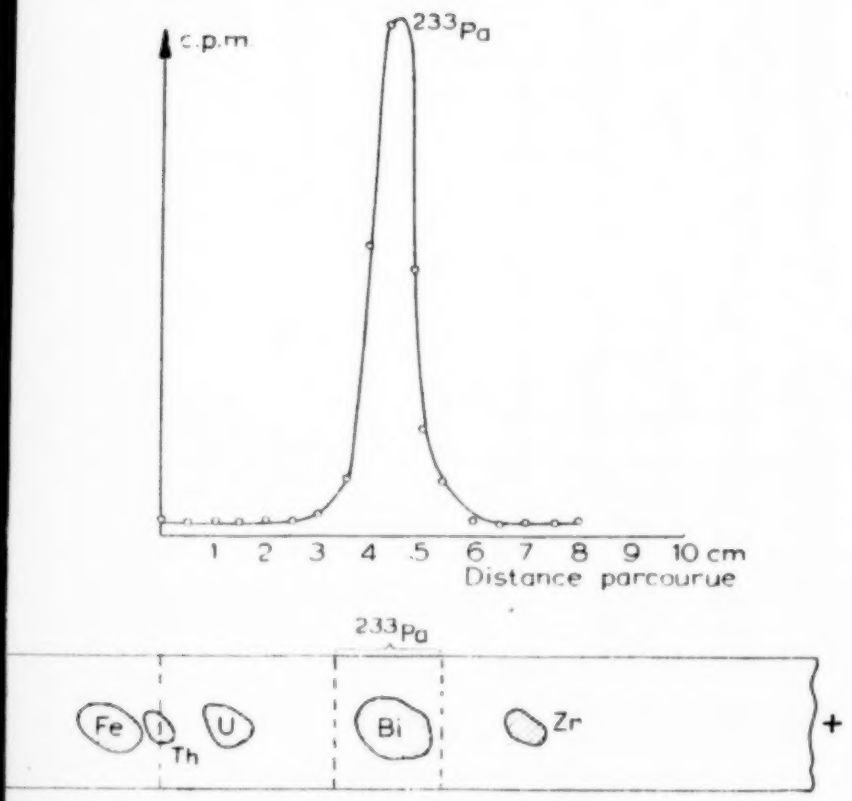

Fig. 4. HCl 0.6 N–HF 2N; voltage 150 V; durée 2 h.

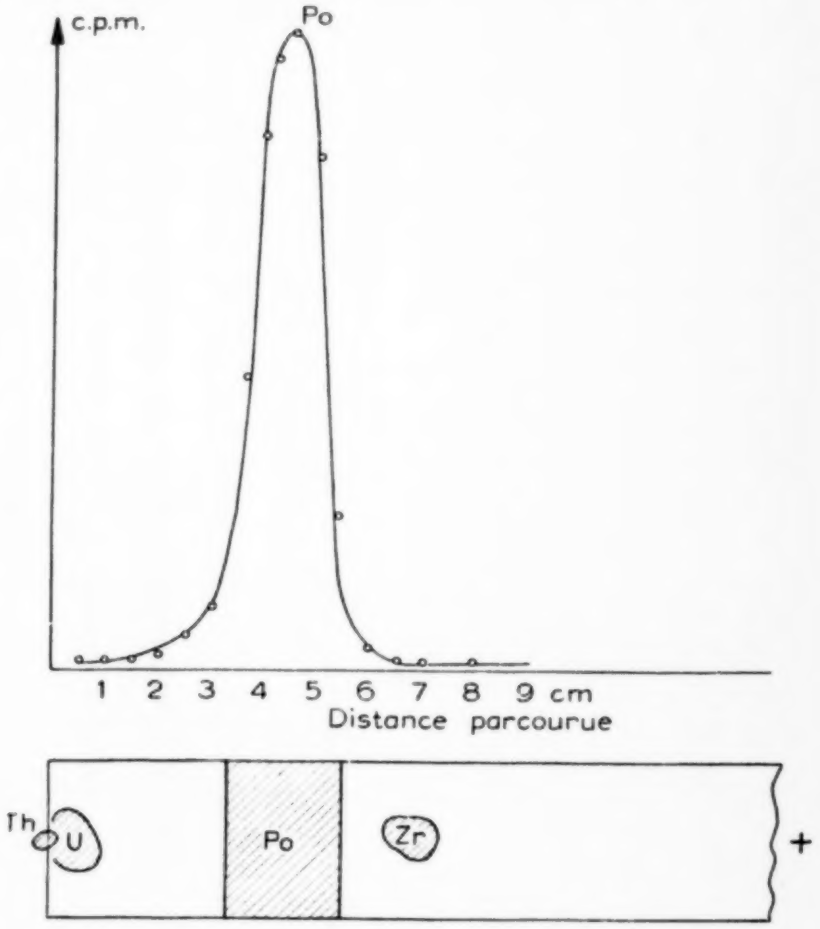

Fig. 5. HCl 0.6 N–HF 2N; voltage 150 V; durée 2 h.

TABLEAU I

MOUVEMENT DES IONS MÉTALLIQUES EN MILIEU HCl 0.6 N–HF 2 N AVEC 150 V PENDANT 2 HEURES

Métal	*Signe de la charge*	*Distance parcourure*
Fe(III)	+	+0.5 cm
U(VI)	—	—0.7 cm
Pb(IV)	0	0
Bi(III)	—	—4.5 cm
Nb(V)	—	—4.5 cm
Ta(V)	—	—4.5 cm
Zr(IV)	—	—6.7 cm
Ti(IV)	—	—6.7 cm
Pa(V)	—	—4.4 cm
Po(III)	—	—4.4 cm

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2. *Électromigration en milieu HF*

Nous avons utilisé des électrolytes contenant de faibles quantités d'HF et modifié légèrement la technique en appliquant, non plus une tache de solution contenant les ions, mais une bande. Les Figs. 6 et 7 montent les migrations de Fe, ^{233}Pa et Zr dans les conditions respectives suivantes.

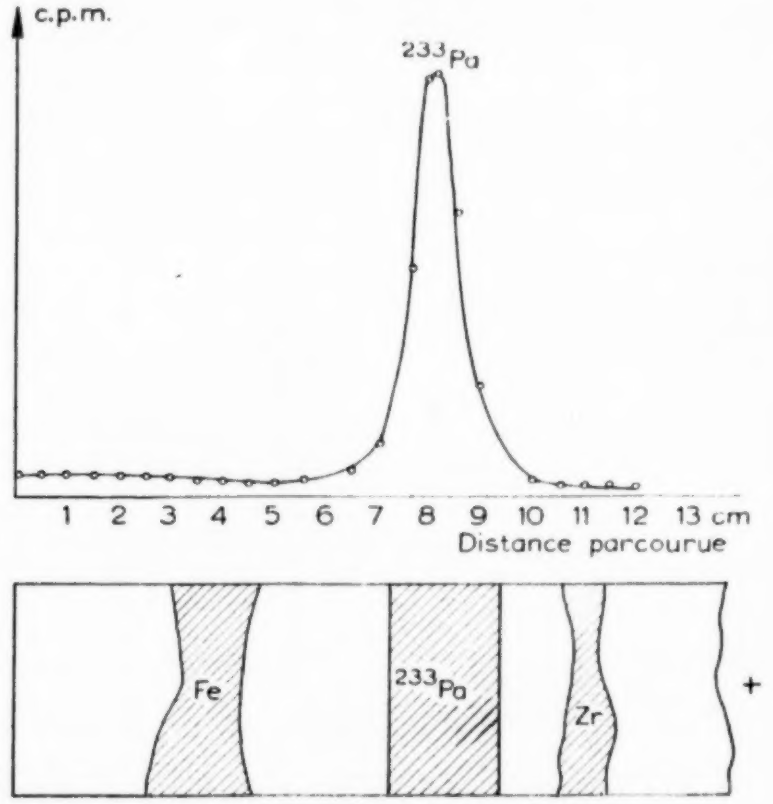

Fig. 6. HF 0.2 N; Voltage 150V; durée 1 h.

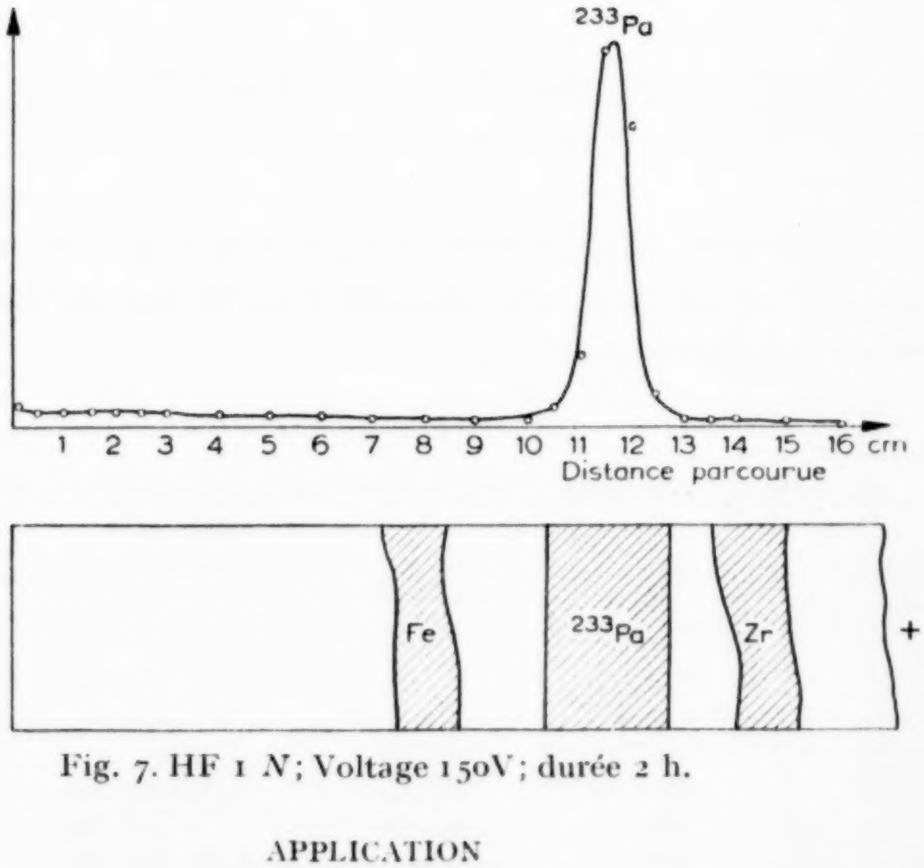

Fig. 7. HF 1 N; Voltage 150V; durée 2 h.

APPLICATION

L'étude précédente nous montre que, dans ces conditions, le protactinium ne peut-être séparé du tantale, du niobium, du polonium ni du bismuth car ces éléments ont des vitesses de migration trop semblables pour espérer réaliser une séparation.

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Cependant de nombreuses séparations intéressant directement la chimie du protactinium ont pu être réalisées.

1. *Séparation Pa–Zr*

Nous avons déjà mentionné la difficulté que présente la séparation du protactinium et du zirconium. Celle-ci peut facilement être réalisée par électrophorèse en milieu HF ou HCl–HF comme le montrent les Figs. 4 et 6.

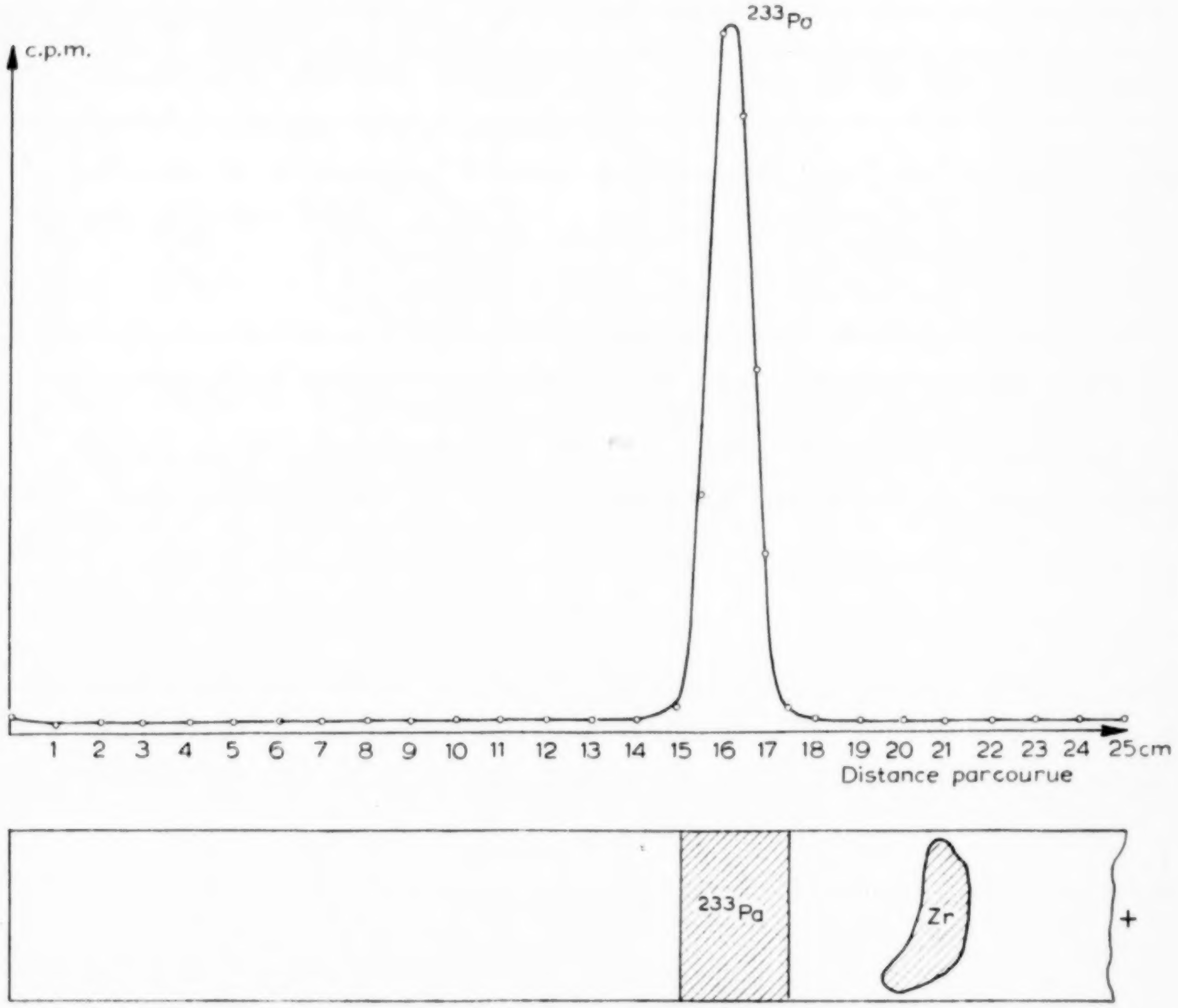

Fig. 8. Séparation Zr–^{233}Pa. HCl 0.6 *N*–HF 1 *N*; voltage 150 V; durée 4 h.

D'autre part, à partir d'un mélange artificiel, nous avons amélioré la séparation en portant la durée de l'électrophorèse à 4 h. L'électrolyte étant constitué par le mélange HCl 0.6 *N*–HF *N*. Cette séparation est representée par la Fig. 8.

2. *Séparation Pa–Ti*

Le titane ayant une mobilité différente de celle du protactinium, la séparation pourra être effectuée en milieu HCl–HF comme le montre la Fig. 3.

3. *Séparation Th–Pa*

La séparation Th–Pa est obtenue soit en milieu HF, soit en milieu HCl–HF. Dans ces conditions, le thorium est précipité au point de départ par suite de l'insolubilité de son fluorure. La manipulation étant assez rapide (de l'ordre de 2 h) cette technique pourrait s'appliquer à la séparation de UX_1 et UX_2 dont les périodes sont respectivement de 24 jours et de 6.7 h.

4. *Séparation Zr–Nb*

En milieu HCl 0.6 *N*–HF *N*, on peut facilement séparer le zirconium du niobium, comme le montre la Fig. 4. Si la durée de l'électrophorèse est portée à 4 h, on obtient une séparation très nette comme le montre la Fig. 9. Cette méthode permet de séparer les deux isotopes radioactifs ^{95}Nb–^{95}Zr, ^{95}Nb étant le descendant direct de ^{95}Zr (Fig. 10).

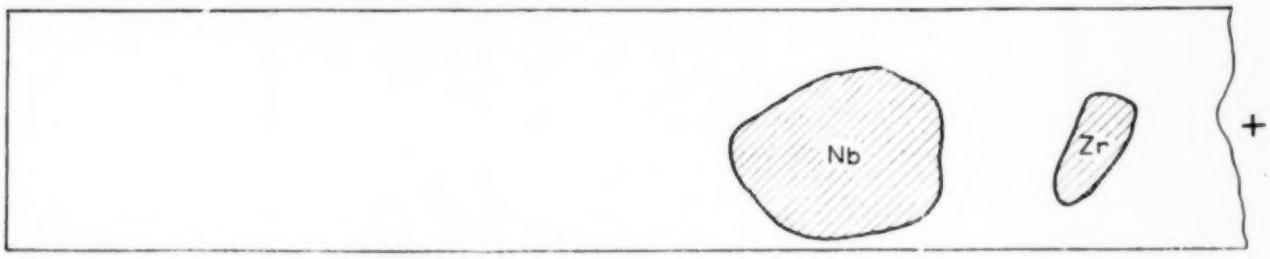

Fig. 9. Séparation Zr–Nb inactif. HCl 0.6 *N*–HF 1 *N*; voltage 150 V; durée 4 h.

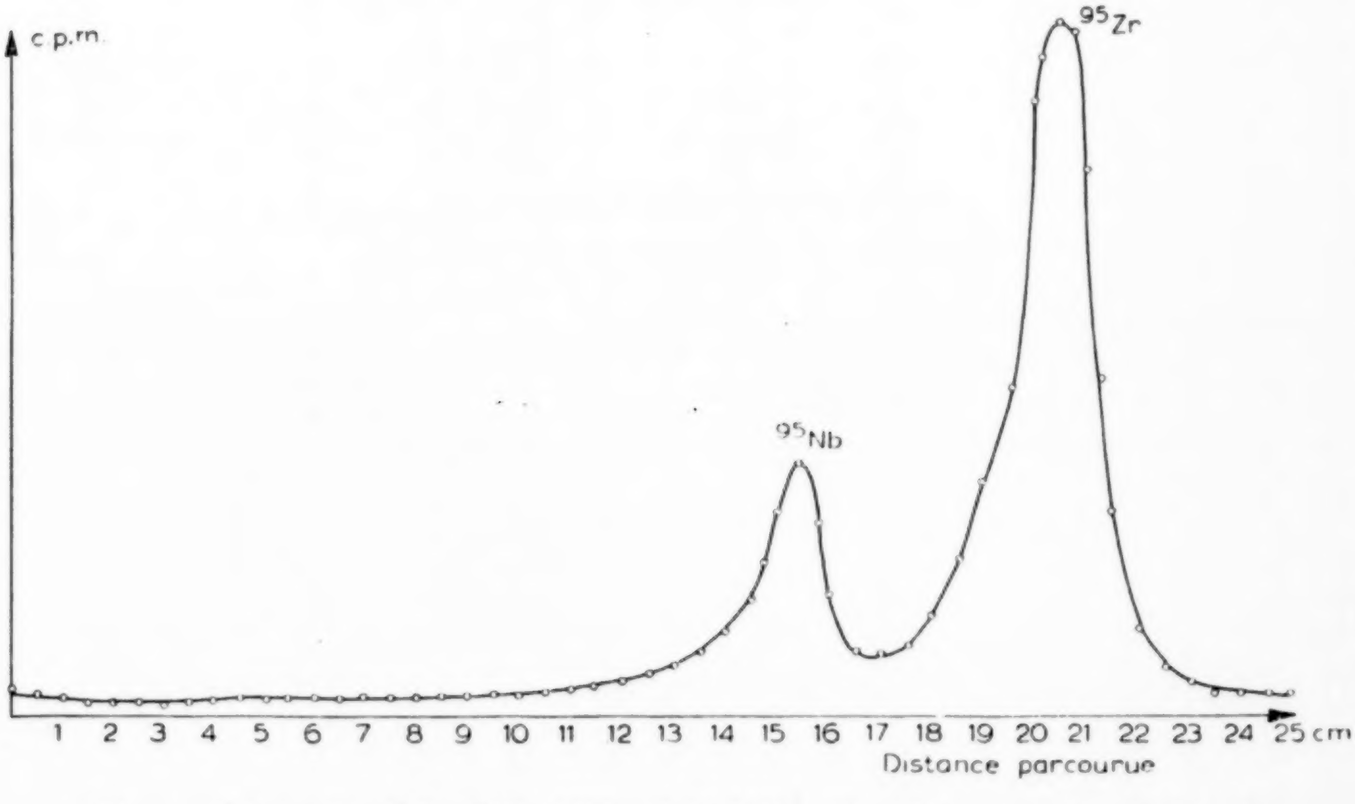

Fig. 10. Séparation ^{95}Zr–^{95}Nb. HCl 0.6 *N*–HF 1 *N*; voltage 150 V; durée 4 h.

5. *Séparation Fe–Pa*

Nous avons déjà souligné l'importance de la séparation Fe–Pa. En milieu HCl–HF ou HF, on peut facilement réaliser cette séparation comme le montre les Figs. 6 et 7.

CONCLUSION: NATURE DES COMPLEXES

Cette étude nous a permis de tirer quelques conclusions quant à la nature des complexes mis en jeu dans les milieux envisagés précédemment.

D'après la séquence des ions, il est probable que le protactinium migrant moins rapidement que le titane et le zirconium, existe dans les milieux envisagés sous la forme PaF_6^-, alors que Ti et Zr seraient sous la forme TiF_6^{--} et ZrF_6^{--}.

La faible distance parcourue par le fer peut s'expliquer par un équilibre entre la forme neutre et deux formes cationique et anionique.

De même pour l'urane

$$FeX^{++} \rightleftharpoons FeX_2^+ \rightleftharpoons FeX_3^0$$

$$UO_2X^+ \rightarrow UO_2X_2^0 \rightarrow UO_2X_3^-$$

X désignant un atome de Cl ou de F.

Le tantale et le niobium, migrant avec la même vitesse que le protactinium existeraient sous la forme NbF_6^- et TaF_6^-.

Le polonium et le bismuth, formant préférentiellement des complexes chlorés seraient sous la forme $PoCl_4^-$ et $BiCl_4^-$.

RÉSUMÉ

Nous avons étudié le comportement électrophorétique de Pa(V), Fe(III), U(VI), Pb(IV), Bi(III), Zr(IV), Ta(V), Nb(V), Th(IV), Ti(IV) et Po(III) en milieux HCl et HCl–HF. Dans ces conditions, le protactinium peut être séparé de certains éléments précités.

SUMMARY

The electrophoretic behaviour of Pa(V), Fe(III), U(VI), Pb(IV), Bi(III), Zr(IV), Ta(V), Nb(V), Th(IV), Ti(IV) et Po(III) in HCl and HCl–HF was studied. In these media protactinium can be separated from some of the above-mentioned elements.

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SEPARATION AND IDENTIFICATION OF PTERIDINES BY PAPER CHROMATOGRAPHY*

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During our attempts to elucidate the pathways of enzymic oxidation of pteridines, it became necessary to separate mixtures that contain isomers as well as pteridines in different stages of hydroxylation[1]. Paper-chromatographic analysis has been successfully applied to pterines, extracted from biological sources[2-6]. Systematic investigations regarding the suitability of various solvent mixtures for the separation of pterines have been carried out by TSCHESCHE AND KORTE[7,8]. Since all naturally occurring pterines have an amino group at position 2, the solvents selected by previous investigators were of special value for the identification of *basic* pteridines. ALBERT *et al.*[9-12] used R_F values extensively for the characterisation of their synthetic pteridines, including hydroxy derivatives devoid of amino groups. Some of the figures obtained by these authors are included in Table II. However, the methods adopted were not chosen for the purpose of analysing mixtures of closely related compounds, such as occur in the course of enzymic oxidation. Especially when there are no side-chains attached to the heterocyclic nucleus and the only difference consists in the number or position of hydroxyl groups, the problem of quantitative separation becomes much more intricate.

In the present study, we have tried to develop new methods for the chromatographic separation and analysis of pteridines, with two main objectives in mind: (a) The procedure should concentrate the compounds tested in well-defined spots. This point is of great importance, in view of the fact that in many solvents certain pteridines tend to streak, while other derivatives may give more than a single spot. The former phenomenon, the tendency to trail, may be ascribed to the presence at each point of equilibrated mixtures of molecular and ionised forms[13]. The latter observation, *viz.* the appearance of more than one circumscribed spot, may be explained by the formation of hydrates (by addition of water to a reactive double bond) that are in equilibrium with the non-hydrated structure. Naturally, if the two members of such a pair are rapidly interconvertible, it would not be easy to understand how they could separate during chromatography. Therefore one is led to assume that during chromatography, conditions are unfavourable for equilibration so that the

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[*] This investigation forms part of the Ph. D. thesis of H. KWIETNY, submitted to the Faculty of Science, The Hebrew University, Jerusalem.

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mixture of hydrated and non-hydrated molecules may behave as though two independent entities were present. Finally, there are indications for keto–enol tautomerism in the pteridine series (SCHOU[14]). If this should prove to be the case, it could lead to the appearance of 2 spots, if the tautomers do not equilibrate easily during development of the chromatogram. (b) The method should be of wide applicability, *i.e.* it should be useful for the separation of a large number of pteridines when present together.

MATERIALS AND METHODS

Substances

The pteridines used in the present investigation were synthesised by ALBERT *et al.*[9–12] and were obtained through their courtesy. The purity of these compounds was checked by ultraviolet spectrophotometry and by paper chromatography. Only for 2,6,7-trihydroxypteridine has the absorption spectrum not been reported previously. An aqueous solution of this derivative apparently consists of a mixture of two different structures[12]; during chromatography it separated into two well-defined spots. However, the synthetic material is undoubtedly pure, because it proved identical in every respect with the substance resulting from enzymic oxidation of 6,7-dihydroxypteridine[1]. At pH 8, 2,6,7-trihydroxypteridine from either source exhibited maxima at 353 and 232 mμ. Although this spectrum is probably not characteristic for a single structure, it is very useful for identification purposes*.

Determination of dissociation constants

The pK values of the compounds used were determined spectrophotometrically, by observing the changes of λ_{max} as a function of pH[15]. The results are unequivocal in all cases in which a steady function is obtained. However, with some 6-substituted pteridines, notably 6-Pt, 2,6- and 6,7-Pt, the number and the position of the absorption maxima change suddenly in a certain pH range, so that an exact evaluation of the pK values by this procedure proved impossible. In these cases, the figures of ALBERT *et al.*, who applied potentiometric methods, were used. However, it should be recalled that the pK values of most derivatives, containing a 6-hydroxyl group, are ambiguous; they depend on the direction of the titration. Inspection of Table I reveals certain discrepancies between our own measurements and those of the Australian investigators. These differences can be ascribed to the different procedures used. In the spectrophotometric method, the substance is dissolved in a given buffer, where it may equilibrate between all possible structures, and then its λ_{max} is measured. In the potentiometric procedure, the pH of a given solution is changed continuously and there may not always be sufficient time for equilibration to take place. Table I demonstrates that most pteridines are very weak bases; they are converted into

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* For the sake of brevity, the pteridines will be designated as follows: 4-hydroxypteridine = 4-Pt; 2,4-dihydroxypteridine = 2,4-Pt; 4,6,7-trihydroxypteridine = 4,6,7-Pt, etc. All mono hydroxy derivatives are classified as group I, all dihydroxy derivatives as group II and all trihydroxypteridines as group III.

TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF PTERIDINES

For examination of fluorescence or of staining properties, 10–20 γ of a pteridine were chromatographed with solvent 6, with the exception of pteridine and 2-Pt. For these two substances solvent 2 was used instead (see Table II). After developing for 12 h, the paper was air-dried and then sprayed with a 0.1% solution of copper acetate in 95% ethanol. After drying, a second spray was applied with 0.5% diphenylcarbazide in 95% ethanol. When solvent 2 was used for development, the paper became impregnated with ammonium chloride and therefore stained red-brown with the above reagents. The spots of pteridine and 2-Pt, which were first detected by observation of their fluorescence, could not be recognised against the background, even when 50 γ of material were used.

Substance	*pK*						*Fluorescence**	*Color of complex with* Cu^{++} *and diphenylcarbazide*
	Albert *et al.*[9–12]			*Our determinations*				
	Cation	*Mono-anion*	*Bis-anion*	*Cation*	*Mono-anion*	*Bis-anion*		
Pteridine	4.1	12.2		3.5	12.5**		Violet	?
Group I								
2-Pt	$<$ 2.0	11.3		~ -2.0	$>$ 12.0		Greenish	?
4-Pt	$<$ 1.5	7.9		$\sim$ 1.0	7.9		Blue	Orange
6-Pt	3.7	6.7					Black-violet	(weakly orange)
7-Pt	-2.0 $+1.2$	6.4		0.5	5.9		Blue-violet	Orange
Group II								
2,4-Pt	$<$ 1.0	7.9		~ -2.0	7.2	12.5	Greenish	Yellow-orange
2,7-Pt		5.8	10.1		5.3	(11.5?)	Sky blue	Red-orange
4,7-Pt		6.1	9.6	~ -2.0	$\sim$ 5.5	$>$ 9	Blue-Violet	Red-orange
2,6-Pt		6.7	11.6	2.0	9.5***		Green	None
4,6-Pt		6.1	9.7	0.7	6.6	9.7	Blue	(Very faint)
6,7-Pt	$<$ 2.7	6.9	10.0				Violet	None
Group III								
2,4,7-Pt		3.6			(3.0?)	9.8	Blue-violet	Dark red-violet
2,4,6-Pt		5.7	9.4	-2.0	5.2	9.6	Blue-green****	(Very faint)
4,6,7-Pt				-2.0	6.7	9.5	Blue	(Very faint)
2,6,7-Pt		3.5	6.7	0.5	6.6	9.1	Upper spot: black-violet Lower spot: sky blue****	None None
2,4,6,7-Pt					$\sim$ 3.5	$\sim$ 9.5	Blue	None

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* It should be noted that the color of the spots under ultraviolet irradiation depends not only on the concentration of the substances, but also on the pH of the solvent used for chromatography. The colors reported are observed when the solvent contained ammonia.

** The absorption maximum of pteridine remains constant at 298 mμ between pH 4 and 12. Above pH 12, it jumps to 320 mμ, indicating ionization due to hydration. This change has been reported previously by Lister, Ramage and Coates[20] and Albert, Brown and Wood[21].

*** The p*K* of 9.5 for 2,6-Pt is a pseudo constant, characteristic for the equilibrium mixture of 2,6-Pt and its hydrate. The true ionization constants of Albert *et al.* belong to a single species only, because they are determined before equilibration can take place.

**** 2,6,7-Pt can often be seen on paper chromatograms with the naked eye as a blue-violet spot. 2,4,6-Pt is always directly visible by its blue-green cations at high acid concentrations. The hydroxy derivatives form anions, the pK decreasing in general with the number of hydroxyl groups present. However, a limit is set by formation of bis-anions— an observation analogous to previous experience in the purine series[15].

Detection of spots

The ability of pteridines to form metal chelates has been studied by ALBERT[16]. Taking advantage of this property, we have tried to stain the chromatograms by spraying the paper first with a solution of a metal acetate and then with a staining agent, specific for the cation used. Among the more stable metal complexes, the Zn^{++} and the Cu^{++} chelates were the most promising. The former, after spraying with an alcoholic solution of diphenylcarbazone, gave intensely red spots. However, since the background was also stained red, only quantities above 50 γ could be recognised unequivocally. Copper acetate and diphenylcarbazide, on the other hand, produced pink to red spots on a bluish-gray background; spots representing 10 γ could be recognised at once and became still more distinct after 24 hours when the background had faded. This proves that the complex pteridine–Cu^{++}–diphenylcarbazide is much more stable than the complex formed in the absence of pteridines. The results of the staining experiments with copper ion are also included in Table I. This detection method is, however, of limited value, since only certain derivatives give a positive reaction. Pteridines containing a 6-hydroxyl group produce only a weak color or may not stain at all. Pteridine and 2-Pt could not be tested, since they concentrate in well-defined spots only in solvents containing ammonium chloride. Chloride ions were, however, found to destroy these complexes. In contrast to the staining procedure, fluorescence in ultraviolet light is of general applicability and was therefore used in all experiments. For this purpose a Mineralight ultraviolet lamp, which emits radiation of about 255 mμ was used.

Chromatography

All experiments were carried out by the descending method, using Whatman paper No. 1. The paper was cleaned by immersion in 0.05 M borax (pH about 9) for one hour and then in 10% acetic acid for the same period. Development extended usually over 12 hours, with the solvent front 35–40 cm from the starting line. In those cases, where the low rate of migration required developing periods of 100 hours (group III in solvent 6 or 8, see Table II) so that the solvent was dripping off the edge of the paper, R_F values were determined by simultaneously running 2,4,7-Pt as reference substance.

RESULTS

Separation of pteridines with various solvent mixtures

The first solvent tested was 3% ammonium chloride (= solvent 1), which has been introduced for pterine studies by TSCHESCHE AND KORTE[7]. As shown in Table II,

TABLE II

R_F VALUES OF PTERIDINES IN VARIOUS SOLVENTS

Substance	*Solvent No.*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Pteridine	streaks	0.87	diffuse	(0.75) diffuse	diffuse	(0.44)	0.84	diffuse	0.25
Group I									
2-Pt	0.37	0.89	0.66	(0.83) diffuse	diffuse	0.47	0.84	0.23	0.8
4-Pt	0.6	0.88	0.44	0.67	0.48	0.5	0.87	0.46	0.5
6-Pt	0.57	0.81	0.66	0.78	0.63	0.53	0.83	0.49	0.3–0.7 (streaks)
7-Pt	0.58	0.83	0.49	0.68	0.64	0.57	0.87	0.49	0.75
Group II									
2,4-Pt	0.56 0.36	0.86	0.37	0.64	0.54	0.5	0.80	0.22	0.5
2,7-Pt	0.48	0.71	0.32	0.51	0.35	0.43	0.77	0.13	
4,7-Pt	0.52	0.79	0.24	0.53	0.35	0.21	0.86	0.24	0.3
2,6-Pt	streaks	streaks	diffuse	streaks	diffuse	0.12	0.75*	0.11	2 spots
4,6-Pt	0.52 0.4 0.19	0.75	0.26	diffuse	diffuse	0.17	0.82	0.15	0.5
6,7-Pt	0.48	0.68	0.25	diffuse	0.45	0.22	0.79	0.13	0.35
Group III									
2,4,7-Pt	0.45	0.63	0.3	0.4	0.2	0.32	0.77	0.13	
2,4,6-Pt	0.5	0.7	0.21	0.5	0.43	0.12	0.76	0.07	
4,6,7-Pt	0.47	0.61	0.17	diffuse	0.13	0.07	0.75	0.035	0.05
2,6,7-Pt	0.69 0.47	0.96 0.62	0.1**	diffuse	0.17**	0.17 0.035	0.85 0.7	0.03**	
2,4,6,7-Pt	0.34 (diffuse)	0.5	0.12	diffuse	0.06	0.06	0.67	0.025	0.05

* Most of the material accumulated in a "head" with green fluorescence. A long tail with faint, blue fluorescence was also observed.

** A second spot was not detected.

Solvent 1: 3% NH_4Cl in water.
Solvent 2: 3% NH_4Cl + 5% NH_3 in water.
Solvent 3: 65 ml isopropanol + 25 ml DMF + 10 ml water.
Solvent 4: 65 ml isopropanol + 25 ml DMF + 10 ml glacial acetic acid.
Solvent 5: 65 ml isopropanol + 22.5 ml DMF + 2.5 ml 90% formic acid + 10 ml water.
Solvent 6: 65 ml isopropanol + 25 ml DMF + 10 ml 25% ammonia.
Solvent 7: 50 ml 95% ethanol + 50 ml 5% ammonia.
Solvent 8: 80 ml 95% ethanol + 20 ml 12.5% ammonia.
Solvent 9: 67 ml *n*-butanol + 33 ml of 5 *N* acetic acid (ALBERT *et al.*[9]).

column 2, all members of group I, with the sole exception of 2-Pt, have about the same R_F. Within group II, the R_F values of all isomers are again close to each other. However, 2,4-Pt gave two spots and 4,6-Pt even three. The R_F of 2,6-Pt could not be measured, because the compound streaks over the whole chromatogram. In group III likewise little variation is observed. All its members develop well-defined, single spots, with the exception of 2,6,7-Pt, which gives two spots in accordance with the experience of ALBERT, LISTER AND PEDERSEN[12]. An important feature is the sharp separation of group III as a whole from 2,4,6,7-Pt, one of the end-products of enzymic oxidation[1]. To summarise: with solvent I there is little differentiation between individual compounds or groups of isomers.

The pH of solvent I was usually between 5 and 6. It is evident from the pK values in Table I that in this pH range most pteridines exist as neutral molecules or as mixtures of the latter with anions. It appeared therefore possible that separation might improve, if all substances were present as anions only. The effect of addition of 5% ammonia to solvent I was therefore studied (solvent 2, pH 10.5; column 3 in Table II). Curiously enough, all R_F values increased considerably. Otherwise, the results with solvent 2 were similar to those with solvent I. In group I, it is remarkable that 2-Pt now behaves like its isomers. In group II, 4,6-Pt is concentrated into a single spot, whereas 2,4-Pt still gives two spots. Like in solvent I, 2,6-Pt spreads over a considerable length of the paper. The titration curve of 4,6-Pt shows a hysteresis loop, indicating the formation of a hydrate[11]; however, in alkaline media equilibration is very rapid. Therefore, in solvent 2 this derivative does not give more than a single spot. In contrast, 2,6-Pt at alkaline pH represents a mixture of two neutral molecules and two anions[12] and the slow transition between the different forms makes the formation of a well-defined spot impossible. The behaviour of 2-Pt is very interesting. This derivative is distinguished from its isomers by its exceptionally high pK value (see Table I). In solvent I, where it is present exclusively as the neutral molecule, it migrates *slower* than its isomers, which are in equilibrium with their anionic forms. Usually, the opposite behavior is observed in paper chromatography, ions travel at lower speed than the neutral molecules. However, the reverse relationship holds in general for solvent I and 2, since the mobile phase contains only water and possesses a higher ionic strength than the stationary phase. As compared to solvent I, solvent 2 is somewhat more suitable for group separation and could indeed be used for certain analytical problems arising from enzymic oxidation reactions[1].

In the special case of 6-Pt, ALBERT, BROWN AND CHEESEMAN[10] could eliminate streaking by the use of dimethylformamide (DMF) as solvent. In the light of our experience with solvent I and 2, it was decided to test DMF in three different forms: (a) in combination with neutral solvents only; (b) together with organic acids, and (c) with ammonia.

The combination of 25% DMF with 65% isopropanol and 10% water (= solvent 3) was selected from about 10 different mixtures for a more thorough study. The results in Table II, column 4, show that only pteridine and 2,6-Pt give diffuse spots. Group I appears to be divided into two pairs of substances, the members of each pair having identical R_F values: 2- and 6-Pt migrate considerably faster than the pair 4- and 7-Pt. In group II and III, the R_F values overlap, and similarly no sharp separation is found between group III and 2,4,6,7-Pt. A most remarkable feature is the appearance of only a single spot for 2,6,7-Pt, a phenomenon observed also with solvent 5. Summarising, it can be said that solvent 3 does not provide the desired solution of the present problem and is suitable only for special purposes (*e.g.* for the separation of a mixture of 2- and 4-Pt or of 2,4- and 4,7-Pt).

In solvent 4, DMF (25%) and isopropanol (65%) were combined with 10% glacial acetic acid instead of water. The results in Table II, column 5, demonstrate the inferiority of this mixture. Many compounds produced diffuse spots and 2,6-Pt showed streaking. ALBERT *et al.*[10] used DMF as its azeotrope with formic acid (6%). We examined therefore solvent 5, which contains 2.5% formic acid and 10% water (see Table II, column 6). Although superior to solvent 4, it was still unsatisfactory, because four derivatives gave diffuse spots. However, some new interesting properties came to light: In group I, 4-Pt could easily be separated from 7-Pt, analytically a very valuable feature. In group II, 6,7-Pt can be differentiated from 2,4-Pt on the one hand and from the pair 2,7- and 4,7-Pt on the other. In group III, 2,4,6-Pt differs from its isomers by its high R_F value. This group as a whole is also well separated from tetrahydroxypteridine.

More encouraging results were obtained by the addition of 2.5% ammonia (solvent 6; column 7 in Table II). Here, all hydroxy derivatives gave well-defined spots; only pteridine itself yielded a rather elongated spot. In group II, 4,7-Pt and 6,7-Pt had identical R_F values; all other members could be separated from this pair and from each other. In group III, excellent separation of all isomers is possible. However, in view of the small rate of migration, development has to be prolonged for more than 100 hours. It is also apparent that 4,6,7-Pt can not be distinguished from 2,4,6,7-Pt. This problem can, however, be solved with the aid of solvent 1 or 2. An important property of solvent 6 is the fact that 4,6,7-Pt can easily be distinguished from both spots characteristic for 2,6,7-Pt. This question actually arose in the elucidation of the oxidative pathway of 6,7-Pt[1].

It should be mentioned that an increase in the ammonia concentration did not produce any improvement over solvent 6. On the contrary, some of the derivatives of 6-Pt, which formed well-defined spots in solvent 6, started to trail again, when 5% ammonia was present. Solvent 6 seems to offer good prospects of solving analytical problems and it was used extensively by us in enzymic studies.

In view of the favorable results obtained by addition of ammonia, we also studied the simple combination of ethanol with this base, in the hope of being able to dispense with the use of the toxic DMF. With 50% ethanol and 2.5% ammonia (solvent 7; column 8 in Table II) all pteridines showed rather high R_F values and very little differentiation. The results were in general very similar to those with solvent 2, but now all compounds formed well-defined spots. A much better separation was achieved by decreasing the percentage of water. Solvent 8, which contained 80% ethanol and 2.5% ammonia, led to well-concentrated spots—with the sole exception of pteridine (see Table II, column 9). In group I, 2-Pt exhibited a much smaller R_F value than the other mono-hydroxy derivatives, which all migrated at about the same rate. In group II, two separate sections can be distinguished: 2,4- and 4,7-Pt possess a much higher R_F than all other members, but these were again not clearly differentiated from each other. In group III, good separation can be effected by developing for a prolonged period. Solvent 8 thus revealed properties similar to those of solvent 6 and proved indeed very useful for special analytical purposes.

In Table II, we have also included the results, reported by ALBERT *et al.*[9-12] for a butanol–acetic acid–water mixture. Besides streaking, the appearance of more than one spot and the overlapping of R_F values between different groups makes it difficult in most cases to use this solvent for the separation of mixtures containing pteridines with varying number of hydroxyl groups.

TABLE III

RECOVERY OF PTERIDINES FROM PAPER CHROMATOGRAMS

For these experiments, 80 γ of each pteridine were spotted on the starting line over a length of 8 cm. After development with solvent 6, the spots were marked under ultraviolet light, cut out and extracted with 8 ml of 0.1 *M* phosphate buffer, pH 8.0. The extracts were read in a Beckman Model DU ultraviolet spectrophotometer at the absorption maxima given in column 2 of the Table. Paper blanks of the same size were treated in the same manner, and the optical density of the blank extracts at the relevant wavelengths was subtracted from the readings of the pteridine extracts. The derivatives marked with an asterisk showed—besides their individual $\lambda_{\max}$—an additional peak at 313 mμ. For pteridine and 2-Pt, solvent 2 was used for development, since in solvent 6 these two compounds do not concentrate satisfactorily (see Table II).

Substance	$\lambda_{\max}$ *(mμ) at pH 8.0*	*% Recovery*
Pteridine	298	74
Group I		
2-Pt	310	83
4-Pt	331	83*
6-Pt	289; 358	82
7-Pt	328	77
Group II		
2,4-Pt	270; 328	90*
2,7-Pt	345; 361	56
4,7-Pt	327	78*
2,6-Pt	298	53
4,6-Pt	281; 359	89
6,7-Pt	320; 336	88
Group III		
2,4,7-Pt	275; 328	90
2,4,6-Pt	381	82*
4,6,7-Pt	317; 330	78*
2,6,7-Pt	354	94
2,4,6,7-Pt	290; 332; 346	87

Recovery of pteridines from paper chromatograms

For the evaluation of the results of enzymic reactions, it is of importance to determine individual pteridines quantitatively after their chromatographic separation. We have therefore extracted the spots with 0.1 *M* phosphate buffer of pH 8.0 and have measured the concentrations spectrophotometrically. As shown in Table III, in most cases a recovery of 70–90% was achieved. Notable exceptions are 2,6- and 4,6-Pt, where not much more than half of the amount applied to the paper, was found in the extract. Incomplete recovery of pteridines may be ascribed to two different factors: Some of the compounds are sensitive to air or light, as observed previously by ALBERT *et al.*[9-12] However, some derivatives of 4-Pt, which are characterized by their great stability, developed a new absorption maximum at 313 mμ. These compounds are marked in Table III by an asterisk. No explanation for this phenomenon has been found, since all substances involved form well-defined, single spots, without any indication of the presence of a common contaminant.

The result obtained with 2,6,7-Pt is especially interesting. After chromatography in solvent 6, two spots are obtained. From the lower one ($R_F = 0.035$), almost 80% of the total amount was recovered, exhibiting $\lambda_{\max} = 353$ mμ. The extract from the upper spot ($R_F = 0.17$) did not absorb at all at this wavelength, but showed a new maximum at 270 mμ, which was absent in the original solution. The optical density of the second extract at its maximum was, however, so small, that only a minor part of the total material could have been present in the upper spot. While these results indicate separation of 2,6,7-Pt into two stable entities, we can confirm the observation of ALBERT *et al.*[12] that each component, upon re-chromatographing, again produces a pair of spots.

DISCUSSION

The results given in Table II demonstrate that no single solvent combination is suitable for the separation of all 15 hydroxylated pteridines. However, in actual problems only certain homologs and isomers occur together. Solvent 6 proved most suitable for the differentiation of such mixtures. The combined use of solvents 6, 2 and 8 has enabled us to solve all analytical problems encountered in enzymic reactions[1].

In the pteridine series, the R_F values decrease regularly with an increase in the number of hydroxyl groups. This observation contrasts with the effect of non-polar substituents. For example, it is well-known that in homologous series the stepwise introduction of methyl or methylene groups *increases* the rate of migration[17, 18]. When $\log(1/R_F - 1)$ is plotted as a function of n, the number of identical substituents attached to a fundamental structure, a straight line should be obtained[19]. For methylated homologs this line exhibits a negative slope[18], *i.e.* the free energy of transfer from the stationary to the mobile phase *increases* regularly with each additional methyl group. In Fig. 1 we have plotted the above function for the R_F values, measured in solvent 2, to demonstrate the *positive* slope of the straight line, *i.e.* the *decrement* of ΔF, the free energy of transfer, for each additional hydroxyl group. Since ΔF is related to the distribution coefficient α by the equation $\Delta F = RT \ln \alpha$, the reversed slope in Fig. 1 is simply an expression of the fact that hydroxyl groups increase the affinity of a given structure for the stationary (*i.e.* aqueous) phase.

Fig. 1 also demonstrates that the linear relationship between log $(1/R_F - 1)$

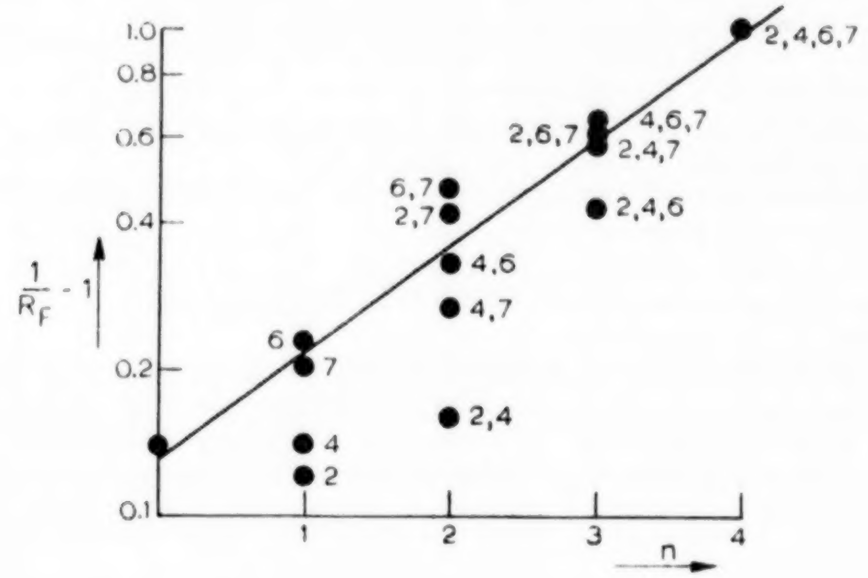

Fig. 1. Log $(1/R_F - 1)$ as a function of n, the number of hydroxyl groups attached to the pteridine nucleus. The values used in this graph refer to solvent 2 in Table II. Numbers indicate the position of the hydroxyl groups. Note the logarithmic scale on the ordinate. The line drawn shows that there is a linear relationship for the following two series: (a) Pteridine → 6-Pt → 4,6-Pt → 4,6,7-Pt → 2,4,6,7-Pt; (b) Pteridine → 7-Pt → 2,7-Pt → 2,6,7-Pt (or 4,6,7-Pt) → 2,4,6,7-Pt.

and n, the number of hydroxyl groups in the pteridine nucleus, holds only for certain derivatives, but is not of general validity. The spread of the R_F values of isomers reveals again the profound influence of the position of hydroxyl groups on the physical properties of pteridines. This influence is analogous to the marked effect of the position of hydroxyls on the chemical behavior of pteridines[10–12].

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SUMMARY

Methods for the paper-chromatographic separation of hydroxylated pteridines have been developed.

The dependence of the R_F values of pteridines on the presence of organic acids or ammonia in the solvents used and on the number and position of hydroxyl groups in the heterocyclic nucleus has been discussed.

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PLANT PHENOLS

I. SEPARATION OF THE TEA LEAF POLYPHENOLS BY CELLULOSE COLUMN CHROMATOGRAPHY

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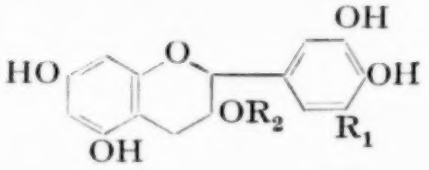
INTRODUCTION

Immediately after plucking, tea leaves are subjected to a series of operations (withering, rolling, fermentation, drying) which convert them to black tea*. The organoleptic characteristics of this manufactured tea depend to a large extent on its polyphenolic constituents, which include the following classes:

A. Flavanols and their gallic acid esters

Six compounds belonging to this class have been isolated from unfermented (green) tea and crystallized (Formulas I to IV). The work in this field has been summarized by ROBERTS[1]; for the configuration of some of these substances see HERGERT AND KURTH[2], ROBERTS[3,4], BIRCH *et al.*[5], FREUDENBERG[6], HARDEGGER *et al.*[7] and BROWN *et al.*[7a].



(I; $R_1 = R_2 = H$)
(+)-Catechin
(—)-Epicatechin

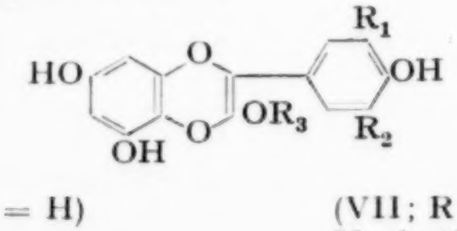
(II; $R_1 = OH$, $R_2 = H$)
(+)-Gallocatechin
(—)-Epigallocatechin

(III; $R_1 = H$, $R_2 =$ galloyl)
(—)-Epicatechin gallate

(IV; $R_1 = OH$, $R_2 =$ galloyl)
(—)-Epigallocatechin gallate

B. Flavonol-3-glucosides

ROBERTS *et al.*[8] have identified 3-glucosides, 3-rhamno-glucosides and 3-rhamnodiglucosides of the flavonols (V) to (VII) in green Assam tea. Numerous flavonol glucosides have also been detected in Japanese teas[9].



(V; $R_1 = R_2 = R_3 = H$)
Kaempferol

(VI; $R_1 = OH$; $R_2 = R_3 = H$)
Quercetin

(VII; $R_1 = R_2 = OH$, $R_3 = H$)
Myricetin

(Va; VIa; VIIa; $R_3 =$ glucoside)
The corresponding 3-glucosides

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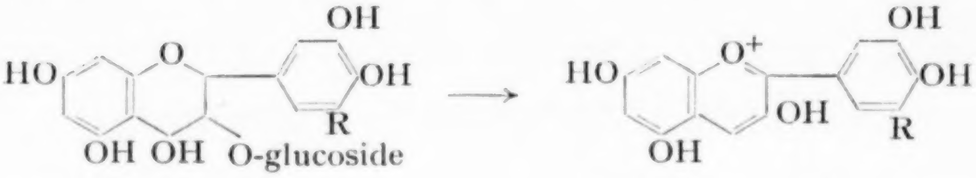
* By "tea leaves" we designate the leaves as they are harvested by the pluckers and by "green tea" the manufactured tea which has been dried without fermentation.

C. Leucoanthocyanins

ROBERTS *et al.*[10] have shown the presence, in green tea, of leucocyanin (VIIIa) and leucodelphinin (IXa), which, upon hydrolysis, yield cyanidin (VIIIb) and delphinidin (IXb).



(VIIIa; R = H)
Leucocyanin

(IXa; R = OH)
Leucodelphinin

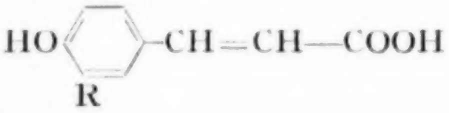
(VIIIb; R = H)
Cyanidin

(IXb; R = OH)
Delphinidin

D. Acids

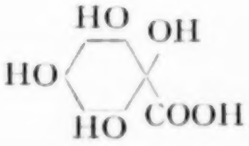
Theogallin (galloyl-quinic acid)[11,12] is found in relatively large quantities, but it has not yet been obtained in crystalline form. All other phenolic acids occur in small quantities.

Gallic acid.
Chlorogenic acid (3-caffeoyl-quinic acid)[13,14].
Neochlorogenic acid (a chlorogenic acid isomer)[14].
p-Coumaryl-quinic acid[14].
m-Digallic acid[11,15].



(X; R = H)
p-Coumaric acid

(XI; R = OH)
Caffeic acid



(XII)
Quinic acid

E. Oxidized and polymerized substances

These compounds, which are present only in small quantities in tea leaves, make up the major polyphenolic part of black tea. They give it its colour and, together with

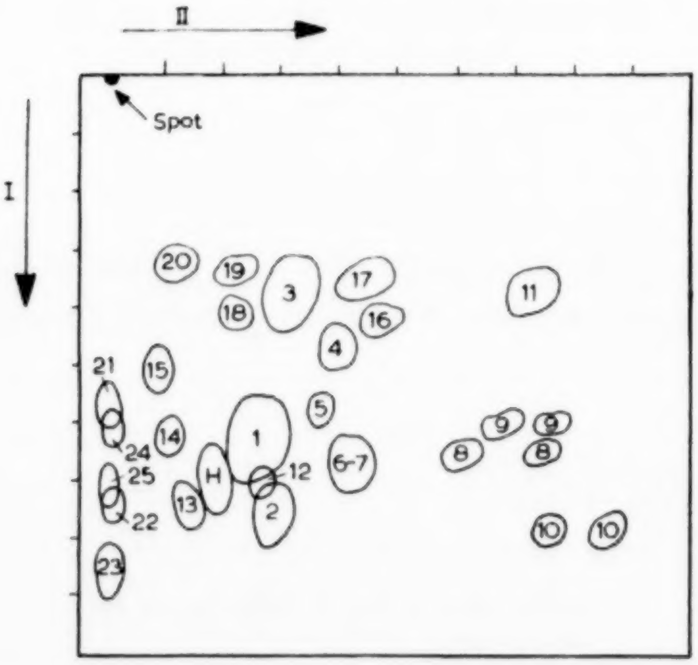


Fig. 1. Paper: Whatman No. 1. Solvent systems for first dimension: butanol–acetic acid–water (4:1:2.2); second dimension: 2% acetic acid in water.

1 (—)-Epigallocatechin gallate
2 (—)-Epicatechin gallate
3 (—)-Epigallocatechin
4 (+)-Gallocatechin
5 (—)-Epicatechin
6 (+)-Catechin
7 Gallic acid
8 Chlorogenic acid
9 Neochlorogenic acid
10 *p*-Coumaryl-quinic acid
11 Theogallin
12 *m*-Digallic acid, according to ROBERTS[11,15]
13 Kaempferol-3-glucoside (astragalin)
14 Quercetin-3-glucoside (isoquercitrin)
15 Myricetin-3-glucoside
16–20 Leucoanthocyanins
21 Myricetin
22 Quercetin
23 Kaempferol
24 Anthocyan I
25 Anthocyan II
H "Substance H"

the volatile aroma constituents, determine its organoleptic properties. They are formed mainly from the flavanols and their gallic acid esters during the manufacture of black tea[1]. Their structure has not yet been elucidated.

Several authors[8, 10, 11, 13–19] have identified the phenolic substances belonging to groups A to D by paper chromatography. Fig. 1 shows a two-dimensional key-chromatogram which is based on our own R_F-values except for *m*-digallic acid[11]. According to WILLIAMS[20], *cis-trans* isomerism could be responsible for the double spotting given by the derivatives of cinnamic acid in the second dimension (aqueous solvent). Table I lists the u.v. fluorescence of the substances present on the key-chromatogram, as well as the colour which develops on spraying with bis-diazotized benzidine.

TABLE I

Substance (spot in Fig. 1)	*U.v. fluorescence*	*Colour with bis-diazotized benzidine*
1– 6	violet	red-brown*
7	blue	yellow turning red-brown*
8– 9	green*	yellow
10	violet*	yellow turning brown
11	blue*	yellow
13–15	purple*	pink*
16–20	violet	brown*
21–23	yellow*	pink-brown
24	pink*	no reaction
25	salmon*	no reaction
H	violet*	yellow

* Indicates the more sensitive reactions.

Tea polyphenols are generally divided into two groups, according to their behaviour upon extraction of an aqueous solution with ethyl acetate:

(a) those passing into the organic phase,

(b) those remaining in the water layer.

The latter are separated from the other constituents of a tea extract (salts, sugars, amino acids, etc.) by precipitation as lead salts.

The composition of the tea leaves used as starting material in our study is given below (percentage calculated for dry leaf weight).

Substances not extractable by 80% ethanol:	%
proteins (Kjeldahl N × 6.25)	15.25
fibers (by difference)	30.25
Substances extractable by 80% ethanol:	
insoluble in water:	
pigments (chloroformic extract minus caffeine)	5.55
soluble in water:	
caffeine (according to *Manuel Suisse des denrées alimentaires*, 1939)	4.65
polyphenols soluble in ethyl acetate	26.70
polyphenols insoluble in ethyl acetate	5.20
amino acids (Kjeldahl N × 6.25)	4.15
ash	4.40
sugars (by difference)	3.85
	100.00

The total polyphenols amount to 66% of the water-soluble substances and the polyphenols extractable with ethyl acetate amount to 83% of the total polyphenols.

The repartition of the polyphenols upon extraction with ethyl acetate depends on the conditions prevailing during extraction of the aqueous phase. Our procedure (see EXPERIMENTAL PART) gave the following results:

1. The flavanols (A) are almost completely extracted.
2. The flavonol glucosides (B) and the leucoanthocyanins (C) are distributed according to the nature of their glucosidic component. As far as the flavonol derivatives are concerned, the 3-glucosides are completely extracted while the others remain in the aqueous phase. It is probable that the same is true for the leucoanthocyanins.
3. As regards the acids (D), at pH 5.5 theogallin remains almost entirely in the aqueous solution while the others are found in both phases.
4. The same applies to the polymerized substances (E), of which only a part is extracted.

Of the polyphenols that are not extracted by the organic solvent, some precipitate completely with lead acetate at pH 5.5 (theogallin, leucoanthocyanins, polymerized substances), others at pH 8.5 (*p*-coumaryl-quinic acid, flavonol-3-glucosides), while the rest (gallic, chlorogenic and neochlorogenic acid) can be found in both precipitates.

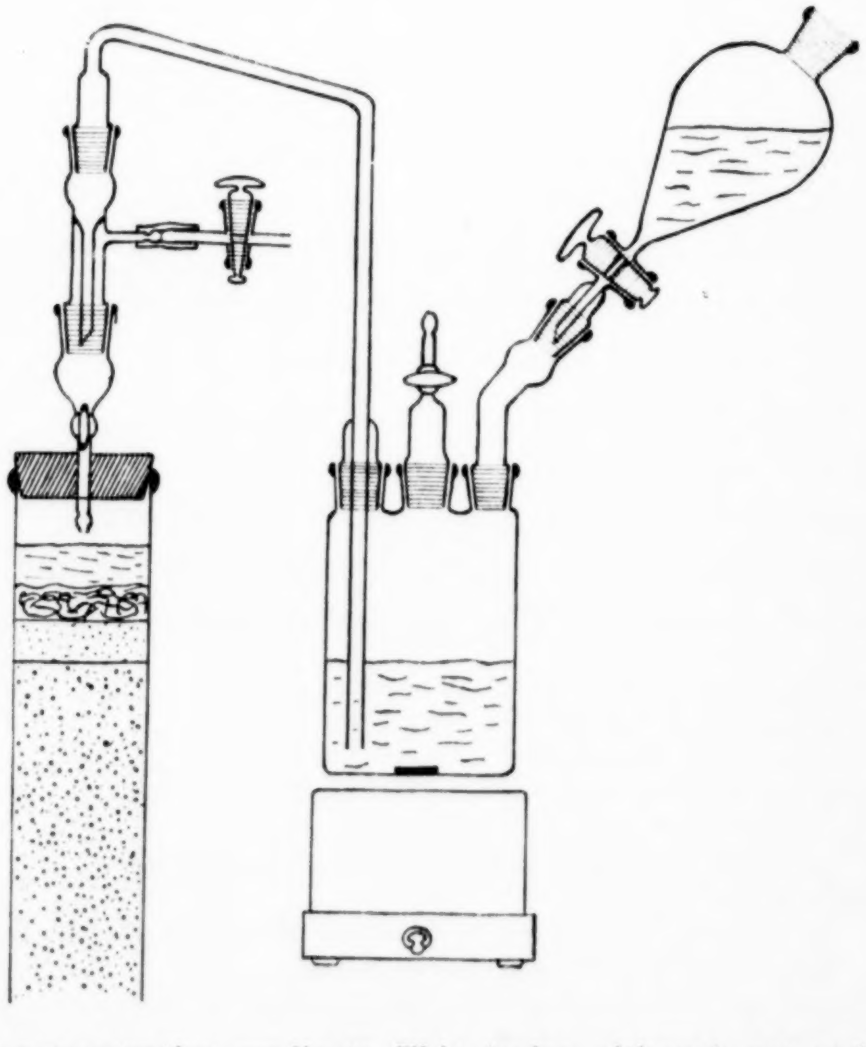


Fig. 2. Device for producing concentration gradients. This device, although not yielding linear concentration gradients as others do[25], has, however, the advantage of greater versatility. By changing the solvent volume in the mixing chamber, the gradient rate may be varied according to need.

For studying the polyphenols of green tea, BRADFIELD *et al.*[21,22] have used partition chromatography on silica gel columns. This technique has also been applied by Russian authors[23]. Furthermore, ROUX[24] has shown that chromatography of these polyphenols from water on a collagen column yields a separation identical to that given in the second dimension of the paper chromatogram described previously.

We have now developed a technique of partition chromatography in which wet cellulose powder is used as stationary phase, and a series of solvents of increasing polarity as mobile phase; these solvents are applied by means of the device sketched in Fig. 2, to yield concentration gradients. The solvents are (in order of application):

1. Ethyl propionate–petroleum ether (9:1), saturated with water.
2. Ethyl propionate, saturated with water.
3. Ethyl acetate, half saturated with water.
4. Butanol, half saturated with water.
5. Methanol, containing 10% water.
6. Water.

The fractions obtained from this partition chromatogram are then further separated by adsorption chromatography from water on a cellulose powder column.

The topic of this first publication is a description of the separation of the tea leaf polyphenols by means of these techniques and their study.

EXPERIMENTAL PART

I. Isolation of polyphenols

(*a*) *Starting material.* Tea leaves, plucked in the fall of 1956 in Ceylon near the coast south of Colombo, were immediately cooled to 4° and shipped by air. They were received in a perfect state of freshness and were extracted 55 hours after leaving the plantation.

(*b*) *Extraction.* The tea leaves (450 g, containing 100 g solids) are homogenized with ethanol (1400 ml ethanol yield, with the leaf moisture, an 80% ethanolic solution). To avoid oxidation, 100 mg $K_2S_2O_5$ as a 10% aqueous solution are added. After desintegration, the mixture is stirred for 20 min at 40°, then filtered. The residue is extracted twice more with 750 ml of 80% ethanol (20 min stirring at 40°). The three filtrates are combined and concentrated to 500 ml in a rotating evaporator under reduced pressure. The caffeine and the pigments which partially precipitate are extracted with $CHCl_3$ using a rotating flask to avoid emulsion formation.

(*c*) *Separation of polyphenol soluble in ethyl acetate.* The aqueous phase is freed of the residual $CHCl_3$ by vacuum evaporation, then extracted six times for 5 min with one-liter volumes of ethyl acetate. The ethyl acetate extracts are combined and concentrated under reduced pressure (CO_2 atmosphere) to 100 ml. 200 ml of H_2O are added, and the remaining ethyl acetate is removed under reduced pressure. The polyphenols are freeze-dried. Yield: 26.7 g of an orange powder.

(*d*) *Separation of polyphenols insoluble in ethyl acetate.* The aqueous phase remaining after ethyl acetate extraction is freed from dissolved organic solvent by vacuum evaporation. A few ml ethanol are added to dissolve the sparingly soluble polyphenols. A saturated solution of $Pb(OAc)_2$ is added.

The precipitate is filtered and washed with 3 l of distilled H_2O with continuous agitation. On gradually adding Dowex 50 (H-form) to the suspension, the Pb^{++} is bound to the cation exchanger and the free polyphenols go into solution. A little ethanol is added to prevent re-precipitation. The mixture is filtered and the resin thoroughly washed with 20% ethanol. The combined filtrates are concentrated under vacuum in a rotating evaporator and the polyphenols freeze-dried. Yield: 3.6 g of a brown powder.

The filtrate and wash water from the Pb-polyphenolates are combined, concentrated under vacuum to 300 ml and adjusted to pH 8.5* with 2 *N* NH_4OH. The yellow precipitate which forms is filtered, washed with distilled H_2O, suspended in methanol and treated with Dowex 50 (H-form). After filtration and washing, the solution is concentrated under vacuum. A small amount of ethanol is added. The resulting white precipitate is discarded and the filtrate is freed from ethanol and freeze-dried. Yield: 1.6 g of a yellow powder.

The total amount of polyphenols precipitated by $Pb(OAc)_2$ is 5.2 g.

II. Adsorption chromatography with water as solvent (for ethyl acetate-soluble polyphenols only)

(*a*) *Preparation of column.* Whatman or Schleicher and Schuell ashless cellulose powder is suspended in distilled water and allowed to settle. The supernatant, containing the colloidal material, is discarded. This process is repeated until the wash-water is clear.

The glass column (68 × 3 cm) is filled with an aqueous suspension of wet cellulose corresponding to 110–130 g of dry powder. The column is packed to obtain a flow rate of 1 ml/min and washed with H_2O until the optical density at 275 mμ ($= E_{275}$) of the effluent is below 0.05 (Beckman Spectrophotometer DU).

(*b*) *Addition of substance.* 1 g ethyl acetate-soluble polyphenols in 4 ml H_2O are placed on the top of the column. After 2–3 washings with a few ml H_2O, the top of the column is fitted with a piece of cotton wool and the elution started.

(*c*) *Elution.* A drop-counter fraction collector (H. Hösli, Bischofszell, Switzerland) was employed. 4 ml fractions are collected. The elution is followed by measuring E_{275} of each fraction.

(*d*) *Analysis of fractions.* The fractions are analysed by two-dimensional paper chromatography on Whatman paper No. 1 with butanol–acetic acid–water (4:1:2.2) in the first dimension and 2% aqueous acetic acid in the second dimension. The spots are detected by their u.v. fluorescence (lamp emitting at 253.5 mμ) and by means of bis-diazotized benzidine[26].

The curve which is obtained by plotting E_{275} against the fraction number, together with the results of the paper chromatographic analysis, permit the individual fractions to be pooled into a few main fractions. These are concentrated under vacuum and freeze-dried.

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* All previous operations were carried out without pH adjustment, that is at a pH of approximately 5.5.

III. Partition chromatography of ethyl acetate-soluble polyphenols

(*a*) *Preparation of column.* Ashless cellulose powder (Whatman or Schleicher and Schuell) was used. The dry cellulose is suspended in the organic solvent and, with vigorous stirring, distilled H_2O (half the weight of the cellulose) is added gradually. The resulting slurry is used to fill the column (68 × 3 cm). Too much water is retained when the column is filled with a suspension of cellulose in water and the latter displaced by an organic solvent. The column is washed until E_{275} of the effluent is below 0.05.

(*b*) *Deposit of substance.* 1 g ethyl acetate-soluble polyphenols are blended with 4 g cellulose powder. The mixture is moistened with a few ml H_2O and placed on the top of the column which is still covered with a small layer of solvent. The preparation is well dispersed (no air bubbles) and allowed to settle before the elution is started.

(*c*) *Elution.* Gradient elution was applied by means of the apparatus sketched in Fig. 2. Ground glass or teflon joints are essential to avoid contamination of the organic solvents. The level of the solvent container is adjusted to permit an effluent flow rate of 0.6–0.7 ml/min. 10-ml fractions are collected and the elution followed as described under IIc. In order of application, the following solvents are used:

1. Ethyl propionate–petroleum ether (9:1), saturated with water.
2. Ethyl propionate, saturated with water.
3. Ethyl acetate, half saturated with water.
4. *n*-Butanol, half saturated with water.
5. Methanol, containing 10% water.
6. Water.

Ethyl propionate (Fluka or Light) was redistilled over a Widmer fractionating column prior to use and the fraction boiling at 98.5° (with E_{275} below 0.05) was employed. The other solvents were Merck reagents for chromatography (purissimum pro analysis). Petroleum ether with the boiling range of 60–80° was used.

(*d*) *Analysis of fractions.* This is carried out as described in IId. The principal fractions are concentrated to a small volume under reduced pressure. A few ml of water are added, the remaining organic solvent is evaporated, and the substances are freeze-dried.

(*e*) *Re-chromatography of principal fractions.* The method described in IIa–d is utilized with a 60 × 1.3 cm column. The optical densities are measured at:

275 mμ for indication of flavanols,
321 mμ for indication of chloro- and neochlorogenic acids,
350 mμ for indication of flavonol glucosides,
380 mμ for indication of oxidized and polymerized substances,
500 mμ for indication of anthocyanins.

Polymerized substances remaining in the column may be eluted by adding ethanol to the water.

IV. Partition chromatography of polyphenols precipitated as lead salts at pH 5.5

(*a*) *Preparation of column.* As described under IIIa, except that the organic solvent is ethyl acetate saturated with water.

(*b*) *Deposit of substances.* As described under IIIb.

(*c*) *Elution.* As described under IIIc, except that both E_{275} and E_{321} are determined, the latter for indicating chloro- and neochlorogenic acids. In order of application, the following solvents are used:

1. Ethyl acetate, saturated with water.
2. Butanol, half saturated with water.
3. Methanol containing 10% water.
4. Water.

(*d*) *Analysis of fractions.* As described under IId and IIId, except that detection is carried out with aqueous 1% $FeCl_3$–1% $K_3Fe(CN)_6$ (1:1).

RESULTS AND DISCUSSION

The data are shown graphically in Figs. 3, 4 and 5.

A. Recovery

1. 82% of the polyphenols applied to the adsorption column are eluted with water (see Fig. 3). The remainder, consisting to a large extent of polymerized substances, may be eluted with ethanol or methanol.

2. The elution of the partition chromatograms (see Fig. 4) is nearly quantitative.

Note: Owing to the different extinction coefficients of the various components, the quantities of the eluted substances are not proportional to the area under the elution curves.

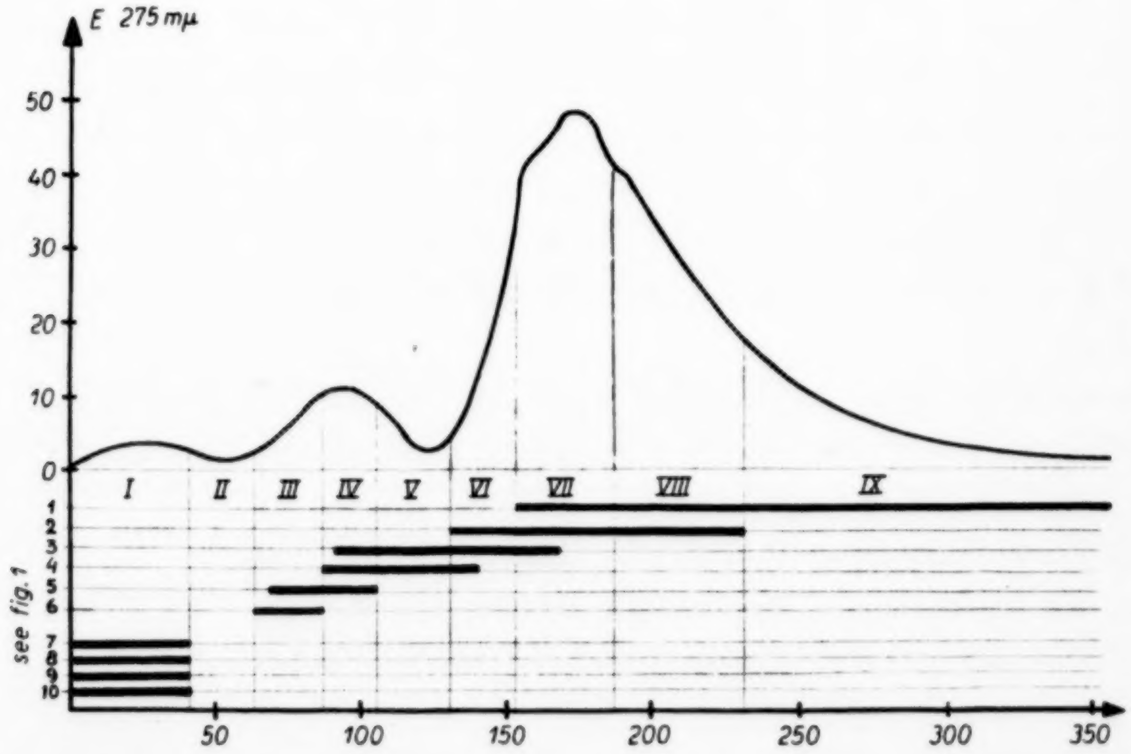

Fig. 3. 1 g polyphenols gives approximately: I–V, 160 mg; VI, 90 mg; VII, 170 mg; VIII, 255 mg; IX, 145 mg; total 820 mg.

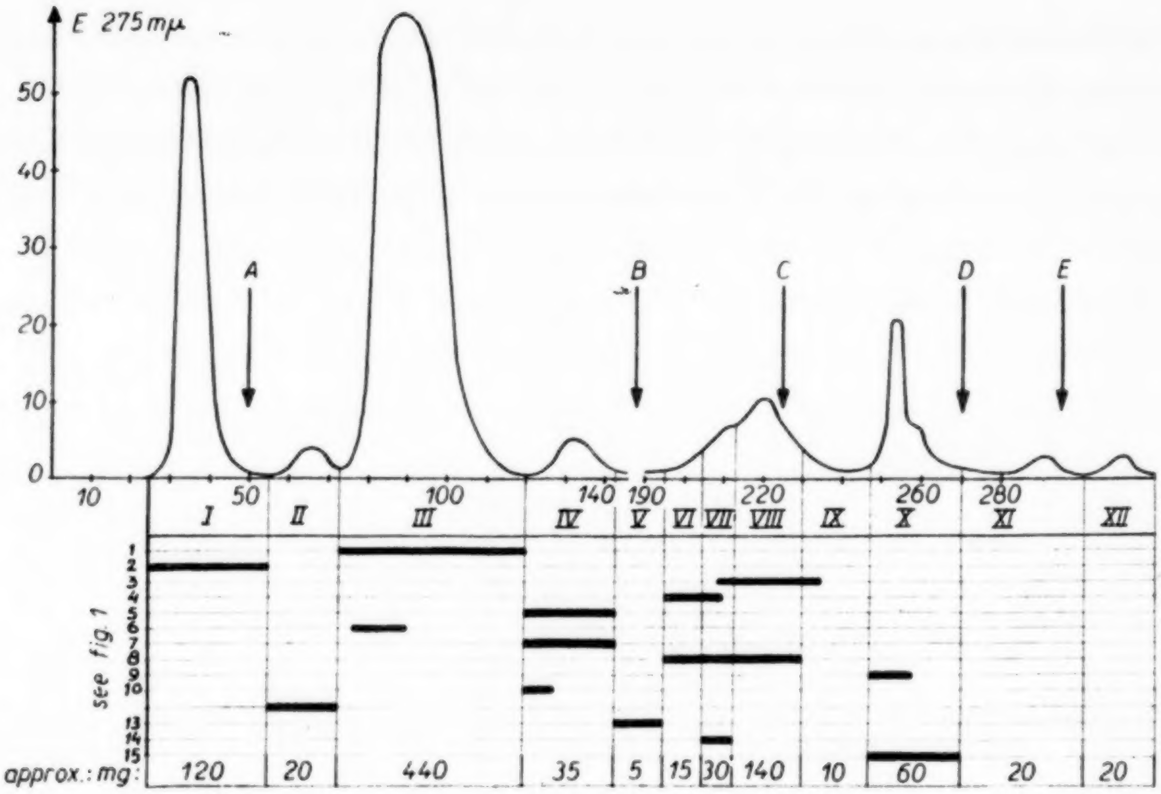

Fig. 4. Beginning of the gradient with: (A) ethyl propionate; (B) ethyl acetate; (C) *n*-butanol; (D) methanol; (E) water.

B. Flavanols

1. Table II shows the R_F values of the flavanols and their order of elution from the column.

In the adsorption chromatogram, the order of elution of (+)-gallocatechin and (—)-epicatechin does not correspond to the R_{FII} values. Inversion also occurs between (+)-catechin and (—)-epigallocatechin gallate in partition chromatography.

TABLE II

Flavanol	R_{FI}	R_{FII}	P	A
(—)-Epigallocatechin gallate	0.64	0.26	2	6
(—)-Epicatechin gallate	0.76	0.28	1	5
(—)-Epigallocatechin	0.37	0.31	6	4
(+)-Gallocatechin	0.48	0.40	5	3
(—)-Epicatechin	0.58	0.37	4	2
(+)-Catechin	0.66	0.41	3	1

R_{FI} = R_F of first dimension } of the two-dimensional paper chromatogram.
R_{FII} = R_F of second dimension }
P = order of elution from partition column.
A = order of elution from adsorption column.

2. With ethyl propionate as eluent, separation of the two gallates is very difficult to achieve. Addition of petroleum ether lowers the distribution coefficient of (—)-epigallocatechin gallate more than that of (—)-epicatechin gallate, thus facilitating their separation.

In the same manner, the distribution coefficient of (+)-catechin is lowered less on addition of petroleum ether than that of (—)-epigallocatechin gallate. With ethyl propionate alone as eluent, (+)-catechin can be found in the tail fraction of the

(—)-epigallocatechin gallate; if the eluent contains 10% petroleum ether, (+)-catechin is eluted in the head fraction of the ester; with 20% petroleum ether, the two substances can even be separated. Nevertheless, we prefer an addition of only 10% and subsequent separation of the two compounds on a small adsorption column, because with higher percentages of petroleum ether elution proceeds too slowly.

3. For analytical purposes, we recommend starting with a separation by partition chromatography in order to eliminate first the two gallic acid esters which predominate quantitatively. Substances present in small amounts can then be detected more easily because they represent a higher relative percentage of the following fractions.

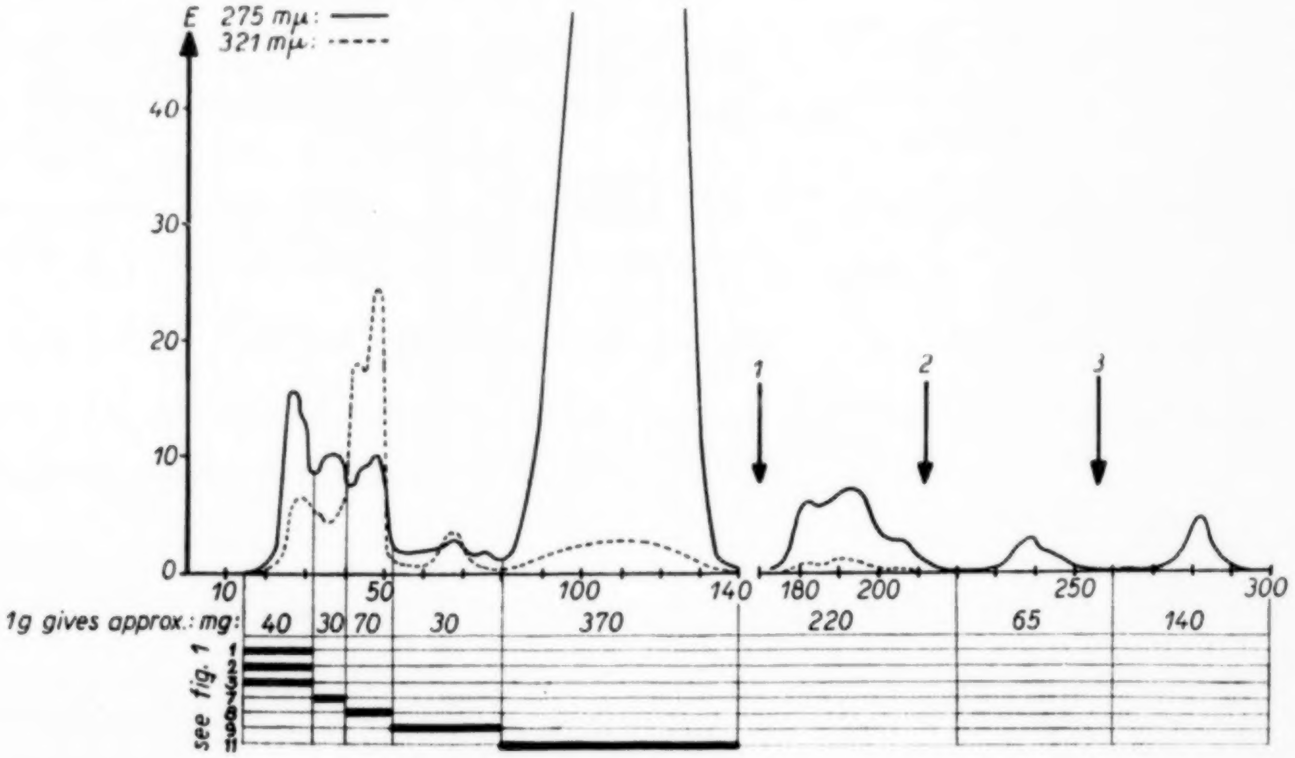

Fig. 5. Beginning of the gradient with: (1) *n*-butanol; (2) methanol; (3) water.

For a preparative separation, we prefer to begin with adsorption chromatography in order to eliminate a large amount of the polymerized substances which are retained on the cellulose. Then it is possible by partition chromatography to isolate from the combined main fractions III to V in a perfectly separated state:

with ethyl propionate:	(+)-catechin and (—)-epicatechin
with ethyl acetate:	(+)-gallocatechin and (—)-epigallocatechin

The combined main fractions VI to IX give:

with ethyl propionate–petroleum ether (9:1):	(—)-epicatechin gallate and (—)-epigallocatechin gallate
with ethyl acetate:	(+)-gallocatechin and (—)-epigallocatechin.

By this procedure, we have obtained the 6 flavanols in their crystalline state.

References p. 187.

4. Analytical data for the crystalline flavanols are reported in Table III.

5. From paper chromatography, ROBERTS AND WOOD[31] concluded that catechin and gallocatechin occur in tea leaf in the (+) form and not in the (±) form as in green tea. Our $[\alpha]_D$ values of the two compounds are a direct confirmation of this assumption.

TABLE III

a. (—)-Epigallocatechin gallate*, crystallized from H_2O.
b. (—)-Epicatechin gallate**, crystallized from $MeOH–CH_2Cl_2$ mixture by gradual addition of CH_2Cl_2.
c. (—)-Epigallocatechin, crystallized from H_2O.
d. (+)-Gallocatechin, crystallized from $AcOEt–CH_2Cl_2$ mixture by gradual addition of CH_2Cl_2.
e. (+)-Catechin***, crystallized from H_2O.
f. (—)-Epicatechin***, crystallized from H_2O.

R_{FI} and R_{FII} are given in Table II, $\lambda_{max.}$ and $\varepsilon_{max.}$ in Table IV. Infrared spectra will be published elsewhere.

	a	*b*	*c*	*d*	*e*	*f*
M.p.						
Literature	215–216°[22] 213°[23]	252–254°[22] 251°[24] 235°[23] 253°[27]	217–218°[21] 212–215°[22] 218°[23] 218°[28]	183°[29]	***	***
Found****	218°	253°	217°	186°		
Formula	$C_{22}H_{18}O_{11}$	$C_{22}H_{18}O_{10}$	$C_{15}H_{14}O_7$	$C_{15}H_{14}O_7$	***	***
Analysis						
%C calc.	57.64	59.73	58.82	58.82		
%C found	57.07	59.32	59.34	58.25		
%H calc.	3.96	4.10	4.61	4.61		
%H found	4.08	4.28	4.68	4.75		
$[\alpha]_D$						
Literature	—179°[22]	—190°[22] —177.5°[27]	—60°[21] —67.5°[28]	+13.1°[29]	+17.1°[30]	—68.2°[30]
Found*****	—185° ±2°	—190° ±2°	—59.5° ±2°	+13.8° ±2°	+13.8° ±2°	—65° ±2°

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* On exposure to air and light, the crystals remain white for 10 days, in contrast to the report of BRADFIELD *et al.*[22] that they readily become brown under these conditions.

** The crystals turn light brown on exposure to air and light for a few days.

*** We did not attempt to obtain (+)-catechin and (—)-epicatechin from tea leaf in an absolutely pure state, because these two substances were available in large amount from other sources. We only compared them by paper chromatography with the pure compounds and measured their $[\alpha]_D$.

**** M.p. measured with Kofler block. Crystals dried over P_2O_5 under high vacuum at room temperature.

***** a, b, c and f: $[\alpha]_D$ determined in ethanol; d and e: $[\alpha]_D$ determined in acetone–water (1:1).

6. BRADFIELD *et al.*[22] have isolated from green tea a flavanol different from the six aformentioned, which they called gallocatechin-a-gallate. ROBERTS *et al.*[31] have shown that the substance appears when an aqueous solution of (—)-epigallocatechin gallate is heated. The transformation which takes place during heating is an

epimerization giving rise to (—)-gallocatechin gallate (BRADFIELD's a-gallate). We did not find this substance in our leaves, which confirms the assumption of ROBERTS that this a-gallate is formed during the manufacture of green tea.

7. The results of the u.v. spectrophotometric investigation of the six flavanols are summarized in Table IV.

TABLE IV

Flavanol	*Lit. values*		*Our values in ethanol*		
	$\lambda_{max.}$	$\varepsilon_{max.}$	$\lambda_{max.}$	$\varepsilon_{max.}$	ε_{275}
(—) Epigallocatechin gallate	275 279.5	9 500[22] 9 250[22]	275	11 500	11 500
(—)-Epicatechin gallate	280	13 600[22]	279	14 000	13 500
(—)-Epigallocatechin	271	1 340[22]	271	1 450	1 275
(+)-Gallocatechin	271	1 734[29]	271	1 460	1 285
(—)-Epicatechin	280	3 300[22]	280	3 580	3 100
(+)-Catechin	280	4 061[29]	280	3 585	3 120

We did not find the double peak for (—)-epigallocatechin gallate reported by BRADFIELD *et al.*[22].

On the basis of the ε_{275} values of the different flavanols and the total density at 275 mμ corresponding to the area under the different peaks (after re-chromatography), we estimate that the following quantities of the flavanols occur in our tea leaves:

	In 1 g ethyl acetate-soluble polyphenols	*% of the dry weight of the leaf*
(—)-Epigallocatechin gallate	390 mg	10.55
(—)-Epicatechin gallate	103	2.75
(—)-Epigallocatechin	88	2.35
(+)-Gallocatechin	14	0.37
(—)-Epicatechin	24	0.63
(+)-Catechin	13	0.35
Total	632 mg	16.90

C. Acids

1. The acids present in the polyphenolic fraction soluble in ethyl acetate (gallic, chlorogenic, neochlorogenic and *p*-coumaryl-quinic acids) are all found in the first peak eluted from the adsorption chromatogram. This is not surprising, for their $R_{F\text{II}}$ values in paper chromatograms with water (containing no acetic acid) as eluent[15] are high.

2. Gallic, chlorogenic and neochlorogenic acids are well separated by partition chromatography. The purification of chlorogenic and neochlorogenic acids from Fractions III and IV (Fig. 5), however, could not be achieved by adsorption re-chromatography. Most of the brown impurities are eluted at the same rate as these acids. A

determination of their quantity, carried out by a new differential spectrophotometric method developed in our laboratories (to be published), indicated that they make up about 45% of Fractions III and IV respectively. A total chlorogenic acid content of approx. 0.3% of the dry leaf weight can be estimated, which include the portion present in the ethyl acetate-soluble fraction.

3. While chlorogenic, neochlorogenic and *p*-coumaryl-quinic acids yield two spots in paper chromatography with an aqueous developing agent, no double peaking could be detected in column chromatography. Chlorogenic acid yields one single peak in a column chromatogram with 2% acetic acid as eluent. Its head, center and tail give the double spotting on paper with the same solvent and it may be concluded that this separation is due to the physical structure of the paper sheet.

4. From paper chromatograms of Fraction V (Fig. 5) it can be assumed that 1 g of the polyphenols precipitated with lead acetate contains about 30% theogallin. Thus, this substance represents approx. 1% of the dry weight of tea leaves. We have not yet been able to obtain it in a crystalline state.

5. The phenolic fraction which does not precipitate with lead acetate at pH 5.5, but at pH 8.5, has been investigated by paper chromatography only. It contains mainly *p*-coumarylquinic acid, accompanied by chlorogenic, neochlorogenic, caffeic, and *p*-coumaric acids (the last two acids probably arise from hydrolysis at this high pH).

D. Anthocyans

ROBERTS *et al.*[10] have found several leucoanthocyanins in Indochina tea leaves, but they do not report having detected anthocyans. The Ceylon leaves used in our investigation contain two substances appearing together as a pink band, which, on partition chromatography, moves very slowly with ethyl acetate, but remains at the top of the column during the entire adsorption run. They may be recovered from the isolated pink section ot the chromatogram by eluting the cellulose with ethanol:

	Anthocyan I	*Anthocyan II*
R_F in butanol saturated with 2 *N* HCl	0.42	0.61
R_F in butanol–acetic acid–water (4 : 1 : 2.2)	0.53	0.69
$\lambda_{max.}$ in ethanol containing 0.01% conc. HCl	510 mμ	495 mμ

Considering our mild extraction conditions, it does not seem probable that hydrolysis of leucoanthocyanins could have occurred. The two anthocyans must, therefore, have been originally present in our tea leaves. (I) might be identical with substance (P) identified by ROBERTS[10a] in black tea.

E. Flavonol glucosides

Adsorption chromatography of partition Fractions V, VII and X (Fig. 4) reveals the flavonol glucosides as yellow bands descending rather slowly. Upon concentrating the eluates, these glucosides crystallize. Hydrolysis with 2 *N* HCl yields the corresponding flavonols myricetin, quercetin and kaempferol, which were detected by paper chromatography.

F. Unidentified substances

1. Paper and column chromatography of tea leaf polyphenols show a number of still unidentified minor substances. For example, chlorogenic acid is often accompanied by compounds giving a blue or violet u.v. fluorescence and yellow reaction with bis-diazotized benzidine.

2. In Fraction II from partition chromatography of ethyl acetate-soluble polyphenols (Fig. 4), the so-called "Substance H" is paramount. Purification of this compound by re-chromatography is difficult, because its affinity to cellulose is almost as great as that of the accompanying polymerized substances. Therefore we have not yet obtained it in a crystalline state. Substance H yields a yellow colour with bis-diazotized benzidine (reaction of acids), a violet u.v. fluorescence (reaction of flavanols), and a violet reaction when sprayed with ferric sulfate (which might indicate a pyrocatechol structure). In ethanol, it exhibits a $\varepsilon_{\max}$ at 279 mμ. The substance is different from gallocatechin-a-gallate, *m*-digallic acid, purpurogallin, and purpurogallincarboxylic acid.

3. On concentrating Fraction IX from an adsorption chromatogram (Fig. 3) to 2 ml, a fine crystalline precipitate appears before the crystallisation of the main component of this fraction, (—)-epigallocatechin gallate. These fine crystals can be separated by centrifugation and washed free from polyphenols with ethanol. They represent approx. 0.2% of the ethyl acetate-soluble tea fraction. The white substance is not polyphenolic, contains neither N or S, does not melt up to 300° (but turns brown at about 250°), and reduces neither Fehling solution nor ammoniacal silver nitrate. The distribution coefficient ethyl acetate/water is near zero, and it is, therefore, to be expected that there is a much larger amount of this substance in the aqueous phase remaining after elimination of polyphenols.

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SUMMARY

The polyphenols of tea leaves have been studied by column chromatography on cellulose powder. Partition and adsorption techniques were applied in succession. In the partition chromatograms, the mobile phase consisted of solvent systems of progressively increasing polarity, and water was used as eluent in the adsorption chromatograms. This technique permits the isolation of nearly all polyphenolic substances of tea leaves.

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PAPER CHROMATOGRAPHY OF AMINO ACIDS AND OTHER ORGANIC COMPOUNDS IN SELECTED SOLVENTS

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The simplicity of apparatus and the ease with which many analyses can be performed have made paper chromatography an outstanding biochemical technique. However, additional information on the characteristic migration rates of compounds in different solvent systems and the influence of variations in temperature and other factors on the R_F values of these compounds is needed to permit ready application of the method in the identification of compounds encountered in routine laboratory work and research. The present paper compares the R_F values of amino acids and other organic compounds in water-saturated phenol, butanol–propionic acid–water, and other selected solvents under controlled and uncontrolled conditions of temperature and humidity. Several compounds for which R_F values are not given in the literature are reported.

The R_F values of several amino acids in phenol have been reported in the literature [1-4]. Though much work has been done with phenol, it is frequently necessary to use two-dimensional chromatography to obtain discrete separation of compounds for identification purposes. The objectional features of collidine and other solvents have been cited by others[5]. A few studies employing butanol–propionic acid–water have been reported[2,6]. However, much of this work has been conducted without adequate control over such conditions as temperature, etc. The use of phenol and butanol–propionic acid–water as solvents in two-dimensional paper chromatography provides a convenient and rapid technique for the separation of compounds in small quantities of biological fluids. The work described herein extends information available on these solvents.

EXPERIMENTAL PROCEDURES

The compounds listed in Tables I and II were applied individually to 18 in. × 22 in. filter paper sheets (Whatman No. 1, especially selected for chromatography) in the upper right corner. The solutions were usually prepared in 50% ethanol in concentrations of 0.5%. 4 λ of a 0.5% solution of Tropaolin 000 No. 1, a dye, was applied 15 mm above the point of application of the sample to facilitate identification when mixtures or solutions containing unknown compounds were studied. Water-saturated phenol, unbuffered, or buffered with 8-quinolinol[7], 25 mg in 500 ml water-saturated phenol, or a solution of 6.3% sodium citrate and 3.7% potassium di-hydrogen phosphate per 100 g phenol[5] was used for the first dimension. The water used to saturate the phenol must be free of all traces of metals[9], therefore glass-distilled water (triple distilled) was used in all instances. A mixture of butanol–propionic acid–water was used for the second dimension[2*].

The papers were run by the descending technique in plywood boxes, 30 in. in height, 19 in. in width, and 34 in. in length. The insides of the boxes were coated with paraffin prior to use to prevent impregnation of the wood by solvents. One chromatocab was always used with phenol, the other with butanol–propionic acid–water. The solvents were contained in pyrex cradles, 24 in. long with a semi-circular cross section 1½ in. in diameter, which rested in stainless steel troughs. Each box was fitted with a 12 in. × 25 in. glass plate on one end to facilitate viewing the papers as the solvent progressed, and was covered with a tightly fitting, felt-stripped lid. Where temperature control is indicated, the chromatocabs were housed in a special room maintained at a temperature of $24 \pm 0.5°$ and at constant humidity. In other instances, the chromatocabs were used in a typical laboratory room where temperature and humidity fluctuated with the weather, though it ranged between 29–35° when the analyses were conducted.

A small quantity of the solvent was placed in a dish at the bottom of the box to bring the atmosphere to equilibrium more quickly with the solvent. 18 to 22 hours were required for migration of phenol down the papers whereas 15 to 16 hours were required for butanol–propionic acid–water. After the phenol run, the papers were dried in a fume hood overnight. They were turned at a 90° angle counterclockwise and butanol–propionic acid–water allowed to descend the papers. They were again dried overnight in a fume hood. Amino acids were located by spraying with ninhydrin (0.2% in ethanol). Color was developed by heating in an air oven at 90° for 5 min. Urea was located by spraying with phenol and sodium hypochlorite, according to the method of BERRY[8]; creatinine was detected with pircic acid[3]; purines were treated with 0.5% nitric acid and ammoniacal silver nitrate[3].

The R_F values (distance travelled by the compound/distance travelled by the solvent) were calculated in the various solvent systems. Following detection, the position occupied by the compounds was encircled with a lead pencil because colors faded on standing over a period of time.

In addition, some compounds were applied to filter paper strips, 1¼ in. wide. The strips were run in selected solvents in a small glass chromatocab, 24 in. high × 12 in. wide, using the descending technique. A tightly fitting glass plate served to cover the glass chromatocab. Ethanol–acetic acid (19:1), 95% ethanol, butanol–ethanol–water (4:1:1), and butanol–acetic acid–water (4:1:5) were used as solvents. The strips were allowed to dry in a fume hood prior to spraying.

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[*] Fresh solvent was prepared from equal volumes of two solutions: A (1246 ml *n*-butanol and 84 ml water) and B (620 ml propionic acid and 790 ml water).

RESULTS AND DISCUSSION

The R_F values for 47 amino acids are given in Table I. The values listed represent, in most cases, the average of two or more runs.

The presence of either 8-quinolinol or the sodium citrate–potassium phosphate buffer affected the migration rates in phenol–water of several of the compounds studied (cysteic acid, cysteine, cystine, glutamic acid, histidine, hydroxyproline, isoleucine, methionine sulfoxide, norvaline, phenylalanine, proline, serine, tryptophan, tyrosine, and valine). Variations in room temperature affected the migration of cysteic acid, glutamic acid, glycine, isoleucine, norleucine, serine, and valine.

Similarly, in butanol–propionic acid–water, the R_F values of arginine, aspartic

TABLE I

R_F VALUES OF AMINO ACIDS AND OTHER SELECTED COMPOUNDS IN PHENOL AND BUTANOL–PROPIONIC ACID–WATER UNDER VARIOUS CONDITIONS

Compound	*R_F value × 100*							*Concentration µg*
	Phenol-water				*Butanol–propionic acid–water*			
	Room temp.	*25°*	*+ 8-Quinolinol 25°*	*+ Buffer 24 ± 0.5°*	*Room temp.*	*25°**	*24 ± 0.5°***	
DL-α-Alanine	63	61	63	61	32	32	32	20
L-Alanine	—	—	—	61	—	—	36	20
DL-α-Amino-*n*-butyric acid	—	—	—	67	—	—	39	20
β-Amino-*n*-butyric acid	—	—	—	81	—	—	40	30
γ-Aminobutyric acid	—	—	—	80	—	—	41	30
α-Aminoisobutyric acid	—	—	—	74	—	—	38	20
β-Aminoisobutyric acid	—	—	—	79	—	—	41	30
α-Aminopimelic acid	—	—	—	91 42***	—	—	52 34***	30
Arginine	55	60	64	64	28	28	19	20
L-Aspartic acid	25	27	32	34	17	24	22	5
Cysteic acid	11	18	16	6	5	9	6	30
Cysteine	—	20	32	20	—	11	—	20
Cystine	20	17	28	14	5	10	8	20
Ethionine	—	—	—	80	—	—	50	20
Ethionine sulfoxide****	—	—	—	80	—	—	30	—
L-Glutamic acid	28	52	41	22	24	32	25	20
Glutathione	—	—	—	32	—	—	5	20
Glycine	37	44	44	40	57	31	20	20
Glycyl-DL-methionine	—	—	—	76	—	—	42	30
Histidine	—	52	76	82	—	24	15	20
DL-Homocysteine	—	—	—	81 39***	—	—	45 20***	20
DL-Homocystine	—	—	—	33	—	—	19	20
Homoserine	—	—	—	64	—	—	32	50
L-Hydroxyproline	65	68	78	64	25	28	21	20
Isoleucine	89	94	93	79	64	68	63	20
Leucine	—	—	—	79	—	—	67	20
Lysine	—	57	53	47	—	19	13	20
D-Methionine	—	—	—	77	—	—	45	30
DL-Methionine	78	81	*****	78	52	57	44	20
DL-Methionine sulfoxide****	—	87	85	76	—	25	24	—

Contd. on p. 191

TABLE I (*continued*)

Compound	*R_F value × 100*							*Concentration µg*
	Phenol–water				*Butanol–propionic acid–water*			
	Room temp.	*25°*	*+ 8-Quinolinol 25°*	*+ Buffer 24 ± 0.5°*	*Room temp.*	*25°**	*24 ± 0.5°***	
L-Methionine	—	—	—	72	—	—	53	20
L-Methionine sulfoxide****	—	—	—	72	—	—	26	—
Methionine sulfone	—	67	67	67	—	25	30	20
Methionine sulfoxide	—	—	—	75	—	—	28	20
Methionine sulfoximine	—	—	—	71 67***	—	—	41 18***	20
Norleucine	95	89	93	88	54	76	71	20
DL-Norvaline	86	87	87	79	47	63	41	20
DL-Ornithine	—	—	—	40	—	—	13	20
Phenylalanine	90	94	91	78	63	67	50	20
Proline	91	95	96	85	37	42	32	20
DL-Sarcosine	—	—	—	72	—	—	27	20
L-Serine	30	42	37	27	18	25	16	20
Taurine	—	—	—	34	—	—	17	20
L-2-Thiolhistidine	—	—	—	25	—	—	15	30
Threonine	48	50	51	46	24	31	25	20
L-Tryptophan	73	75	82	74	54	49	40	20
L-Tyrosine	69	67	63	54	46	45	36	20
Valine	80	87	85	71	57	57	49	20

* Papers were run first in phenol + 8-quinolinol.
** Papers were run first in buffered phenol.
*** Two spots obtained, lower spot.
**** Formed in phenol by oxidation.
***** Completely oxidized to the sulfoxide.

TABLE II

R_F VALUES OF SELECTED COMPOUNDS IN PHENOL AND BUTANOL–PROPIONIC ACID–WATER

Compound	*Quantity µg*	*R_F value × 100**			
		Buffered phenol–water		*Butanol–propionic acid–water*	
		Average	*Range*	*Average*	*Range*
Purines					
Adenine	50	87	85–89	57	51–64
Hypoxanthine	50	90	87–92	38	36–39
Uric acid	50	21	20–22	21	20–21
Xanthine	50	48	45–51	32	31–33
Miscellaneous					
Allantoin	50	55	54–56	27	26–28
4-Amino-5-imidazole carboxamide	30	89	—	39	—
Creatine	50	91	91–92	37	35–40
Creatinine	20	90	—	48	—
Cystathionine	50	26 18**	—	14 8**	13–15 7–9**
Dimethylaminoethanol	5	79	—	38	—
Ethanolamine	0.5	76	74–79	40	39–41
Glutaric acid	75	68	—	72	—
Urea	60	68	—	49	—

* At 24 ± 0.5°.
** Two spots obtained, lower spot.

acid, glutamic acid, glycine, histidine, hydroxyproline, lysine, DL-methionine, norleucine, norvaline, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were different when temperature was not controlled in contrast to being controlled.

The R_F values for L-methionine were higher than those for either D- or DL-methionine in butanol–propionic acid–water. R_F values for other selected compounds in phenol–water and butanol–propionic acid–water are given in Table II.

Chromatography of methionine with other compounds

Methionine was chromatographed with other selected amino acids because it was often necessary in our work to identify this amino acid in the presence of other compounds which migrated to approximately the same position. In such instances, a solution of the compounds was prepared in 50% ethanol, and aliquots of this were applied to 18 in. × 22 in. filter paper sheets or 1 ¼ in. wide filter paper strips.

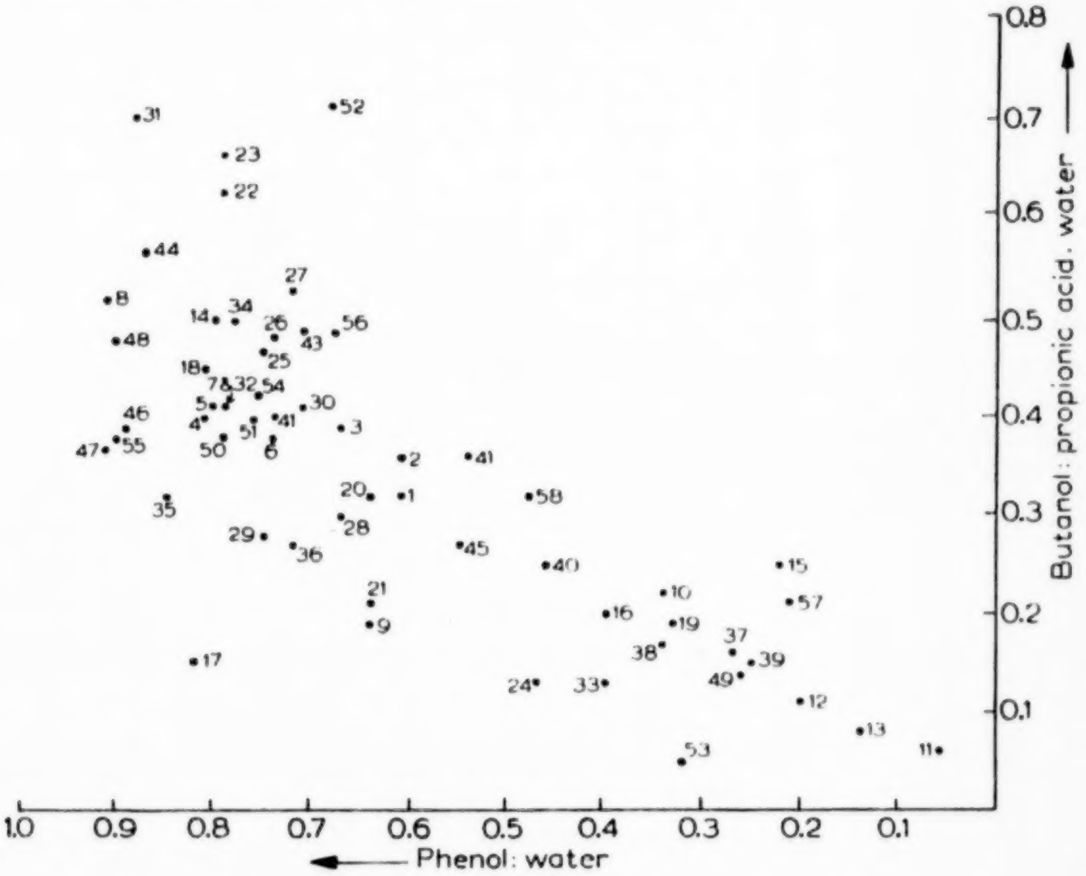

Fig. 1. Map of R_F values of amino acids and other selected organic compounds. 1. DL-α-Alanine; 2. L-Alanine; 3. DL-α-Amino-*n*-butyric acid; 4. β-Amino-*n*-butyric acid; 5. γ-Aminobutyric acid; 6. α-Aminoisobutyric acid; 7. β-Aminoisobutyric acid; 8. α-Aminopimelic acid; 9. Arginine; 10. L-Aspartic acid; 11. Cysteic acid; 12. Cysteine; 13. Cystine; 14. Ethionine; 15. L-Glutamic acid; 16. Glycine; 17. Histidine; 18. DL-Homocysteine; 19. DL-Homocystine; 20. Homoserine; 21. L-Hydroxyproline; 22. Isoleucine; 23. Leucine; 24. Lysine; 25. D-Methionine; 26. DL-Methionine; 27. L-Methionine; 28. Methionine sulfone; 29. Methionine sulfoxide; 30. Methionine sulfoximine; 31. Norleucine; 32. DL-Norvaline; 33. DL-Ornithine; 34. Phenylalanine; 35. Proline; 36. DL-Sarcosine; 37. L-Serine; 38. Taurine; 39. L-2-Thiolhistidine; 40. Threonine; 41. L-Tryptophan; 42. L-Tyrosine; 43. Valine; 44. Adenine; 45. Allantoin; 46. 4-Amino-5-imidiazole carboxamide; 47. Creatine; 48. Creatinine; 49. Cystathionine; 50. Dimethylaminoethanol; 51. Ethanolamine; 52. Glutaric acid; 53. Glutathione; 54. Glycyl-DL-methionine; 55. Hypoxanthine; 56. Urea; 57. Uric acid; 58. Xanthine.

These data are shown in Table III.

A comparison of the data in Table III with those in Tables I and II reveals that

TABLE III

EFFECT OF OTHER COMPOUNDS ON THE R_F VALUE OF METHIONINE

Compounds chromatographed with L-methionine	*R_F values × 100**			
	Buffered phenol–water		*Butanol–propionic acid–water*	
	L-Methionine	*Other compound*	*L-Methionine*	*Other compound*
α-Aminoisobutyric acid	77	74	52	43
Allantoin	82	52	46	25
Arginine	81	64	48	—
Creatinine	74	90	51	56
Ethionine	75	78	59	68
Histidine	79	77	46	20
Leucine	75	82	47	57
Leucine and		85		56
isoleucine	80	85	47	56
Methionine sulfone	77	60	41	22
Methionine sulfone and		60		22
sulfoxide	79	78	41	22
Methionine sulfoxide	78	77	42	23
Norleucine	76	83	47	47
Norvaline and		79		49
urea	81	80	53	50
Phenylalanine	77	81	55	63
Phenylalanine,		83		53
urea, and		91		52
creatinine	77	77	46	49
Proline	74	85	47	38
Tryptophan	75	75	48	50
Valine	77	78	48	48

* Run at 24 ± 0.5°.

the presence of other compounds in the applied solution did not affect R_F values in phenol–water as much as the presence of buffers in the solvent or variations in temperature. The presence of other compounds along with methionine, however, appeared to influence the R_F values of methionine in butanol–propionic acid–water (methionine sulfone, methionine sulfoxide, methionine sulfone and sulfoxide, tryptophan, arginine, valine, ethionine, leucine and isoleucine, histidine, norleucine, leucine, proline, phenylalanine, urea and creatinine, and allantoin).

Both the R_F values for methionine and the R_F values for methionine sulfoxide, tryptophan, arginine, ethionine, norleucine, leucine, and proline were affected when these compounds were chromatographed together in pairs or in groups of three in butanol–propionic acid–water. However, though the R_F value for methionine was influenced by creatinine and phenylalanine, R_F values of the latter compounds were not affected when they were run with methionine.

Effect of group chromatography on the R_F values of individual compounds

It was observed early in our work that R_F values of individual compounds may differ slightly when chromatographed in the presence of several other compounds. Accordingly, this influence was evaluated in the two solvent systems. Solutions of selected amino acids were applied to filter paper sheets in the manner described previously.

TABLE IV

EFFECT OF GROUP CHROMATOGRAPHY ON THE R_F VALUES OF AMINO ACIDS AND OTHER COMPOUNDS

Compound	*R_F value × 100**			
	Buffered phenol–water		*Butanol–propionic acid–water*	
	*Average***	*Range*	*Average*	*Range*
DL-α-Alanine	57	54–60	30	—
L-Alanine	51	46–57	29	—
DL-α-Amino-*n*-butyric acid	64	54–69	31	—
α-Aminoisobutyric acid	71	67–75	33	—
Arginine	61	59–65	21	—
Cystine	18	—	10	—
Ethionine	80	76–89	51	45–57
L-Glutamic acid	29	23–35	22	—
Glutathione	30	25–32	6	5–6
Glycine	38	36–42	20	—
L-Hydroxyproline	64	58–69	21	20–22
Isoleucine	83	77–89	51	45–56
Leucine	82	79–85	51	45–56
Lysine	50	47–53	15	—
L-Methionine	79	77–83	47	41–51
L-Methionine sulfoxide	77	76–79	26	24–28
Methionine sulfone	60	—	22	—
Methionine sulfoxide	77	73–79	22	—
Norleucine	82	77–89	49	45–52
DL-Norvaline	76	72–79	50	48–54
DL-Ornithine	39	37–40	15	—
Phenylalanine	82	79–89	54	49–58
Proline	86	84–89	32	31–32
DL-Sarcosine	75	72–78	26	25–27
L-Serine	32	29–34	20	19–21
Taurine	39	33–45	15	—
Threonine	48	45–51	22	—
L-Tryptophan	68	67–69	43	37–49
L-Tyrosine	50	47–56	36	35–37
Valine	76	72–79	48	—
Allantoin	52	49–55	25	—
Creatinine	92	88–95	63	52–67
Urea	76	70–80	50	45–51

* Run at 24 ± 0.5°.
** Average of 2–7 values.

Solution A contained threonine, tyrosine, arginine, α-amino-*n*-butyric acid, lysine, glycine, glutamic acid, cysteine, aspartic acid, isoleucine, methionine, norleucine, methionine sulfone, ethionine, histidine, methionine sulfoxide, and α-aminoisobutyric acid. Solution B contained proline, hydroxyproline, alanine, taurine, cystine, homocystine, sarcosine, leucine, tryptophan, and phenylalanine. Solution C contained α-alanine, serine, valine, ornithine, norvaline, and glutathione. The values from these three analyses and others in which groups of compounds were chromatographed were averaged and are shown in Table IV.

When several compounds were present simultaneously in the applied solution, the effect on R_F values of individual components appeared to be great. In both phenol–water and butanol–propionic acid–water, R_F values of alanine, aspartic acid, glutamic acid, L-methionine, methionine sulfone, and norleucine were different when

TABLE V

R_F VALUES OF COMPOUNDS IN VARIOUS SOLVENT SYSTEMS

Compounds	*R_F values × 100*[*]			
	Ethanol–acetic acid	*Butanol–acetic acid–water*	*Butanol–ethanol–water*	*Ethanol*
4-Amino-5-imidazole carboxamide	25	—	—	—
Creatinine	42	—	25	—
Ethionine	37	—	35	—
Glycyl-methionine	26	—	—	—
Hydroxyproline	13	—	—	—
Isoleucine	53	61	39	—
Leucine	50	62	37	—
Methionine	28	46	—	—
Phenylalanine	33	—	30	—
Proline	22	—	—	—
Tryptophan	22	56	19	—
Urea	42	70	37	—
Valine	47	48	24	—
Creatinine and	42		27	—
phenylalanine	34	—	32	—
Isoleucine and	52[***]	61[***]	35[***]	—
leucine	52	61	35	—
Methionine and	21[**]	47	26	—
tryptophan	21	52	19	—
Methionine and	28	46	23	—
urea	39	70	32	—
Methionine and	26	47[***]	23[***]	—
valine	40	47	23	—
Phenylalanine and	30	—	31	—
leucine	50		39	—
Phenylalanine and	31[***]	—	31	—
ethionine	31		35	—
Tryptophan and	20		18	—
valine	40	—	25	—
Urea and	37			
tryptophan	19	—	—	—
Urea and	34			
valine	35	—	—	—
Methionine,		46	28	20
creatinine and	—	38	30	28
urea		45	36	38
Valine,		41	30	28
creatinine and	—	34	31	28
urea		41	38	38

[*] Room temperature.
[**] Incomplete separation of spots.
[***] One spot.

chromatographed in the group in contrast to treatment as individual compounds.

The R_F values of DL-α-amino-n-butyric acid, α-amino-isobutyric acid, isoleucine, leucine, methionine sulfoxide, norvaline, and urea were affected only in butanol–propionic acid–water, whereas those for glutamic acid, L-methionine sulfoxide, and tryptophan were different only in phenol–water.

Comparative migration of compounds in different solvents

In several instances, the solvents commonly employed in our work did not cause discrete separation of amino acids and other compounds. Since it was necessary to identify certain substances in the presence of others, R_F values of selected compounds were determined in different solvent systems. Table V presents these data.

It will be noted that, by use of the appropriate solvents, all of the compounds listed in Table V, which migrate to the same positions in phenol–water and butanol–propionic acid–water, with the exception of isoleucine and leucine, can be separated.

Formation of methionine sulfoxide from methionine

It was noted early in our work that two spots appeared on methionine chromatograms when these were run in phenol–water (buffered and unbuffered) and butanol–propionic acid–water systems. From a series of side experiments in which methionine was chromatographed with suspected compounds, it was learned that the "lower spot" was methionine sulfoxide.

Chromatograms were prepared of methionine-2-^{14}C, non-radioactive methionine, methionine sulfoxide, other amino acids whose R_F values were close to the position of the lower spot and various combinations of these. The papers were run in buffered phenol–water and in butanol–propionic acid–water one-dimensionally; and two-dimensionally in both solvents. Autoradiograms were made of the chromatograms containing radioactive methionine.

Autoradiograms of methionine-2-^{14}C presented two areas of radioactivity, corresponding to R_F values of 0.72 and 0.53 in phenol–water and butanol–propionic acid–water, respectively, for methionine and in the position below methionine, 0.72 and 0.26. Subsequent studies were initiated to identify the lower spot.

Radioactive and non-radioactive methionine always presented two spots when run both in phenol–water and butanol–propionic acid–water two-dimensionally. Combinations of methionine and methionine sulfoxide gave only two spots, corresponding exactly to the two positions obtained when methionine was chromatographed alone. The radioactivity in the lower spot from methionine on autoradiograms always coincided with the ninhydrin-positive spot from methionine sulfoxide on chromatograms. This was not true for combinations of methionine with other amino acids. Co-chromatography tests in which the radioactive ninhydrin spots were excised, eluted, concentrated and reapplied with methionine revealed that the methionine lower spot always traveled on chromatograms with methionine sulfoxide.

In one-dimensional runs in phenol–water or in butanol–propionic acid–water, however, only one spot was observed from methionine. In addition, when the two-dimensional chromatograms were run first in butanol–propionic acid–water, and then in phenol–water, only one spot was obtained from methionine.

It was apparent from these findings, therefore, that methionine is oxidized by phenol to methionine sulfoxide, but because the R_F values of methionine and methionine sulfoxide are the same in phenol–water, these compounds appear as one spot on one-dimensional chromatograms. However, because the R_F values for methionine and methionine sulfoxide are different in butanol–propionic acid–water, papers containing methionine run previously in phenol–water show two spots, indicating separation of methionine from its sulfoxide. Similarly, when the solvents are reversed, that is, when

TABLE VI

EFFECT ON R_F VALUES OF CHROMATOGRAPHING AMINO ACIDS IN BUTANOL–PROPIONIC ACID–WATER FIRST, THEN IN PHENOL

Compound	*R_F values × 100**			
	Buffered phenol–water		*Butanol–propionic acid–water*	
	first	*second*	*first*	*second*
α-Aminoisobutyric acid	74	73	38	48
Cystine	14	20	10	11
Glutamine	58 23**	33***	20 23**	19***
Homocysteine	81 39**	22***	45 20**	16***
Homocystine	33	25	19	17
Isoleucine	79	83	63	72
Leucine	79	87	67	76
L-Methionine	72 72**	78***	53 26**	56***
Methionine sulfoximine	71 67**	63***	41 18**	19***
Phenylalanine	78	82	50	66
L-Serine	27	31	16	20
L-Tryptophan	74	75	40	57
L-Tyrosine	54	56	36	39
Valine	71	76	49	56
Creatinine	90	93	48	52
Urea	68	75	49	60

* Run at 24 ± 0.5°.
** Lower spot.
*** Only one spot was obtained with the reversed solvent systems.

papers are run in butanol–propionic acid–water first, and then in phenol, only one spot is present after two-dimensional runs because the sulfoxide is formed in the second run with phenol and does not separate because the R_F values are identical in phenol. This latter point was proved by permitting papers which had already been exposed to the two solvents in reversed order, to run again after a 90° turn in butanol–propionic acid–water. Two spots again appeared.

The development of two spots from methionine in the two-dimensional solvent systems proved extremely helpful in later work in which radioactive methionine was fed to rats and its metabolites were studied by chromatographic techniques.

R_F *values of amino acids in reversed solvent runs*

When amino acids were run first in butanol–propionic acid–water, then in buffered phenol–water the shapes and sizes of the spots revealed after spraying with ninhydrin were different from those run in the usual manner. It was of interest to determine whether the R_F values of the compounds were also changed appreciably. It will be noted from Table VI that values in buffered phenol–water for cystine, leucine, and urea were altered when butanol–propionic acid–water was the first solvent. R_F values in butanol–propionic acid–water for α-aminoisobutyric acid, isoleucine, leucine, phenylalanine, tryptophan, valine, and urea were higher also when this solvent system was used first.

Though several compounds separated into two spots when run in phenol–water first, then in butanol–propionic acid–water, when the solvents were reversed, only one spot was obtained. This would indicate that the second spot was formed from the compound by oxidation with phenol, as in the case of methionine discussed earlier in this paper.

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SUMMARY

The influence of variations in temperature, presence and absence of buffers, and presence of other compounds in the applied solution on the R_F values of 70 organic compounds has been evaluated in phenol–water and butanol–propionic acid–water systems. R_F values of selected compounds in ethanol–acetic acid, butanol–ethanol–water, butanol–acetic acid–water, and ethanol are given. The production of methionine sulfoxide from methionine in phenol–water is discussed.

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USE OF 4,5-DINITROVERATROLE FOR THE ULTRAMICRODETERMINATION OF REDUCING SUGARS ON PAPER CHROMATOGRAMS BY A REFLECTANCE METHOD

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Recently 4,5-dinitroveratrole was suggested as a sensitive qualitative test reagent for reducing sugars[1], and subsequently it was developed into a colorimetric method for the quantitative determination of reducing sugars in the range 0.5–3.0 mg[2]. Preliminary qualitative tests with this reagent showed that 1 μg of fructose, glucose, or arabinose could be detected on paper chromatograms. Further investigation showed that quantitative measurement of 2–20 μg of reducing sugars on paper chromatograms was possible. The experimental procedure and the results are presented.

MATERIALS AND METHODS

Standard solutions of arabinose, glucose, and fructose were prepared, separately, in concentrations of 2, 5, 10, 15, 20, and 25 μg per 5 μl of solution.

The 4,5-dinitroveratrole (4,5-d) was prepared according to the procedure of DRAKE *et al.*[3]. At time of use, as a dip reagent, a solution of 1 g 4,5-d per 100 ml of acetone was prepared.

One mole of potassium hydroxide was dissolved in sufficient 95% ethanol to make one liter of solution. This reagent should be freshly prepared and not stored for future use.

Using a micropipet, 5 μl aliquots of the sugar solutions were applied on Whatman No. 1, chromatographic grade, 18 × 22 inch paper. The chromatograms were irrigated by the descending procedure for 16 h at 25° with a solvent mixture of ethyl acetate–pyridine–water (8:2:1)[4]. After irrigation the chromatograms were removed from the cabinet and air-dried 30–60 min.

The chromatogram was first dipped into the 4,5-d reagent, then allowed to hang for 5–10 min, or longer if necessary, to permit complete volatilization of the acetone. The chromatogram was then dipped into the alcoholic KOH solution and again allowed to hang until the alcohol had completely evaporated from the sheet. The sheets were heated in an oven, in a humid atmosphere, at 60° for 10 min. Immediately after removing the chromatogram from the oven, the reflection densities of the sugar spots were measured with a Photovolt reflectance unit (Model 501-A)[5]* using light of 515 mμ wave length. Each spot was carefully scanned and the maximum density value recorded. The curve of each sugar for each chromatogram was obtained by plotting the amount of sugar (in the logarithmic direction) on semilogarithmic paper against the Photovolt reflection density readings.

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* Mention of manufacturers and commercial products does not imply recommendation by the Department of Agriculture over others of a similar nature not mentioned.

RESULTS AND DISCUSSION

The reflection density readings of the sugar concentrations between 2 and 20 μg/5 μl of solution showed a linear relationship between the logarithm of the sugar concentration and the reflection reading (Fig. 1).

Because of the limitations of the uniformity of the spot densities, multiple spots of each sugar concentration should be applied to a given chromatogram[6,7]. To further minimize differences due to variations from one sheet to another, a separate sugar curve should be prepared from each chromatogram, and the standard sugar curve and unknown sample should always be prepared on the same chromatogram. Adhering to these conditions each point on the curve between 2 and 20 μg can be reproduced within ± 1 μg.

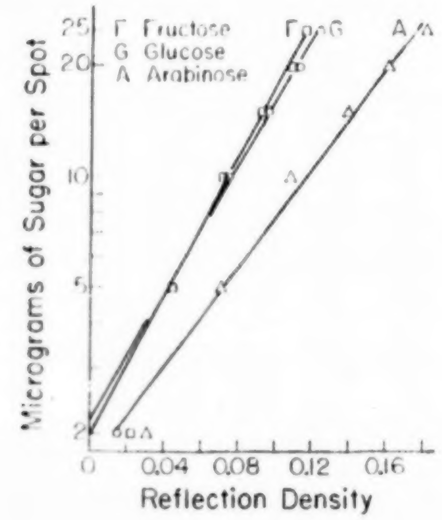

Fig. 1. Standard sugar curves, obtained by plotting amount of sugar, in the log direction on semilog paper, against photo volt reflection density readings using light of 515 mμ wave length.

The 4,5-d will give a qualitative test for reducing sugars at room temperature if the treated chromatogram is allowed to remain for several hours. However, to obtain the maximum color intensity, heat and a moist atmosphere are necessary. Variable time and temperature factors were studied and the optimum conditions were found to be 10 minutes at 60°.

The curves (Fig. 1) produced by the 6-carbon aldose and ketose-type sugars are similar whereas the aldopentose sugar curve has a different slope, primarily because this sugar produces with the 4,5-d reagent a more intensely colored spot of greater area than the aforementioned sugars.

Although numerous methods exist for the quantitative determination of sugars, only a few extend into the ultramicro range, and these do not distinguish an individual sugar in a sugar mixture. Therefore, the use of 4,5-d as a reagent for the determination of microgram quantities of individual sugars, via paper chromatograms, may find application.

SUMMARY

Using 4,5-dinitroveratrole as the chromogenic reagent, an ultramicro method has been developed for the quantitative measurement of 2–20 μg of reducing sugars on paper chromatograms.

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SHORT COMMUNICATIONS

A demonstration of continuous "electrochromatography" and "electrophoresis"

During a study of different supporting media for continuous paper electrophoresis an anomalous migration of a cationic dye (Brilliant Blue) was observed. Whereas the cation migrated towards the negative pole when an electrical field was applied across a glass fiber curtain, the ion exhibited slight anionic movement on cellulose paper. An anionic dye (Amaranth) exhibited anionic movement in both media.

A Spinco-Beckman Model CP Continuous Flow Paper Electrophoresis apparatus was used. Barbital buffer, pH 8.6 at an ionic strength of 0.05 and a constant voltage of 300 V d.c. were employed. The flow of buffer on the vertical edges was regulated so that no migration occurred in the absence of an electrical field[1]. Two water-soluble dyes were selected for this study, Brilliant Blue, the disodium salt of 4-{[4-(N-ethyl-N-*p*-sulfobenzylamino)-phenyl]-(2-sulfoniumphenyl)-methylene}-1-(N-ethyl-N-*p*-sulfobenzyl)-$\Delta^{2,5}$-cyclohexadienimine, and Amaranth, the trisodium salt of 1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid. The dyes were made up as 1% solution in the barbital buffer.

With a cellulose filter paper curtain (Whatman 3 MM), it was observed that both Brilliant Blue and Amaranth (red) migrated towards the anode (Fig. 1) with Brilliant

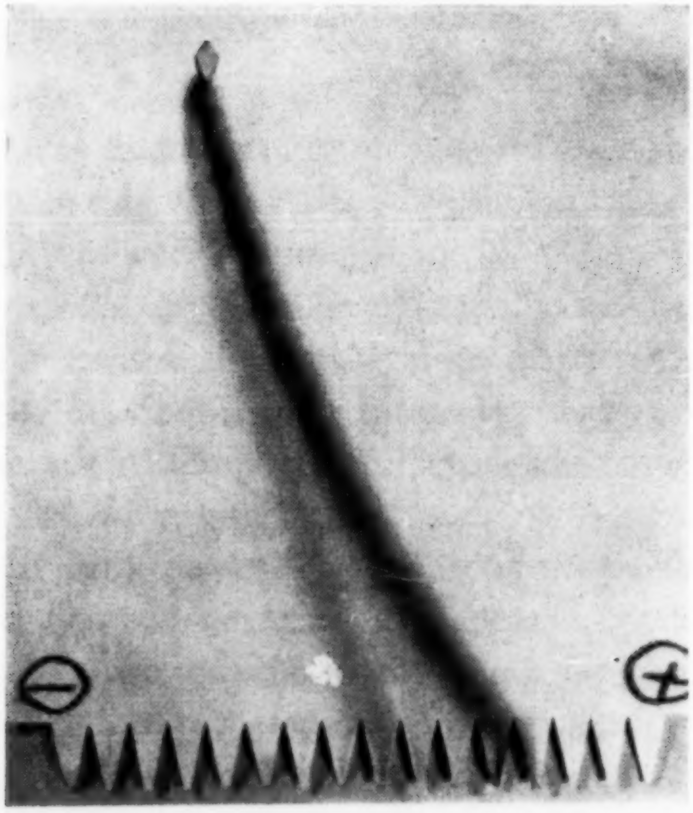

Fig. 1. Separation of dyes on cellulose. Left: Brilliant Blue; right: Amaranth.

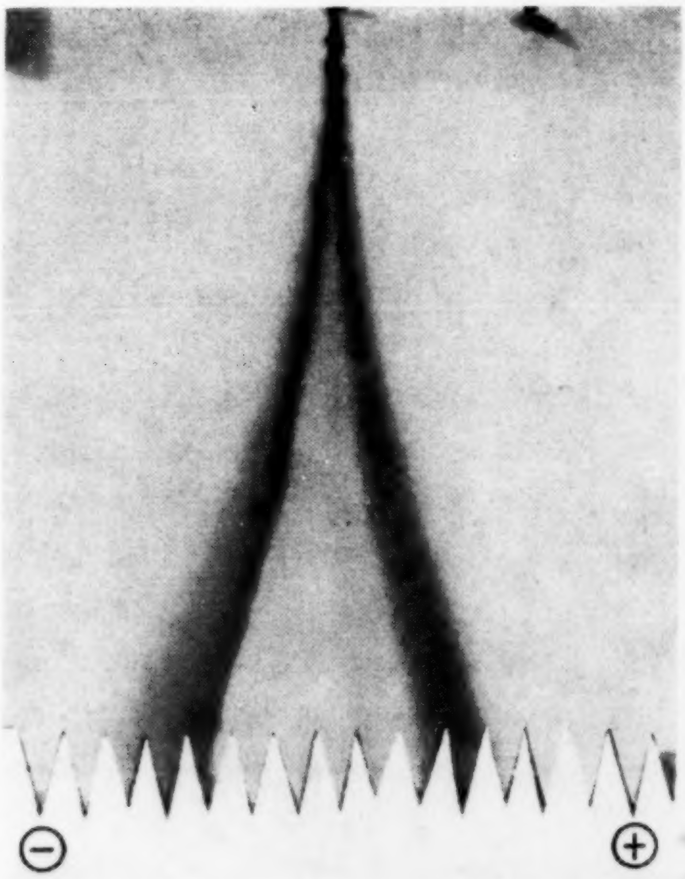

Fig. 2. Separation of dyes on glass. Left: Brilliant Blue; right: Amaranth.

Blue moving at a slower rate. When a glass paper curtain (Reeve Angel glass paper) was substituted for the cellulose, the red dye still moved towards the anode, while Brilliant Blue migrated towards the cathode (Fig. 2). In Table I are listed the numbers of the respective tabs at which the bulk of each dye was collected.

It was also observed that when both dyes were fed onto the cellulose curtain, without the application of an electrical field, Brilliant Blue moved vertically about twice as fast as Amaranth, while on glass fiber both dyes moved at the same speed. This was an indication that Amaranth was more strongly adsorbed on cellulose than Brilliant Blue.

On the basis of these results, it is strongly indicated that the vectors operative in the movement of the cation (Brilliant Blue—a quaternary amine) and the anion (Amaranth—a trisulfonic acid) on glass fiber are the vertical flow of buffer and the transverse electrical field. The resultant migration is "continuous electrophoresis".

TABLE I

	Glass	*Cellulose*
Brilliant Blue	+5	—3
Amaranth	+8	+3

On the other hand, the apparently "anionic" behavior of Brilliant Blue on cellulose paper indicates that other forces are operative, *e.g.* endosmosis. This view is supported by the larger anionic displacement of Amaranth on cellulose than on glass. Even if endosmosis made a small contribution in the migration of Amaranth, one would expect a larger displacement due the adsorption of this dye on cellulose. The use of cellulose filter paper as a supporting medium precludes the term "continuous electrophoresis", and the usage of the term "electrochromatography" becomes more applicable[2].

PUČAR[3], as quoted by STRAIN[4], concluded that migration of sorbed ions on cellulose paper, due to chromatographic and electrical forces, resulted in no greater separation than electrical migration alone in a nonsorptive medium. The anomalous behavior of the cationic dye (Brilliant Blue) on cellulose, as shown here, seems to indicate, however, that sorption in an electrical field and in solvent flow is not proportional, and that other forces (like endosmosis) must be considered.

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The effect of molecular structure on the R_F value
A contribution to the theory of paper chromatography

Paper chromatography is perhaps the most widely used research tool. Unfortunately the choice of the solvents is rather intuitional, since no theory is available which connects the R_F with the molecular structure of the solute.

In the present paper a thermodynamical derivation is presented, which defines the R_F in terms of the molar volume and solubility parameter of the material to be chromatographed and the solubility parameters of the solvents used.

Materials and methods

Whatman No. 1 filter paper was used in all experiments. According to our determinations this paper contained about 30% water when it was equilibrated with water-saturated air at room temperature (determined by measuring the loss in weight of the paper after drying at 100°).

The volume of the moving phase was determined by immersing the tip of a 1 × 10 cm paper strip into the appropriate solvent. After the ascending solvent had wetted the strip entirely, it was weighed in a stoppered test tube. The difference between the weight of the wetted and the non-wetted strip, divided by the density of the solvent, gives the volume of the solvent phase.

Results and discussion

According to MARTIN AND SYNGE[1], paper chromatography is a continuous distribution of the solute between a stationary phase and a mobile phase. By assuming adsorption to be negligible he was able to deduce the following equation, which connects the distribution coefficient α[*] with R_F:

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[*] The following symbols are used throughout this paper:

α = distribution coefficient = $\frac{\text{conc. in stationary phase}}{\text{conc. in mobile phase}}$
A = cross section
$\Delta\mu$ = free energy of transfer from one phase to the other
T' = melting point in absolute degrees
T = temperature in absolute degrees
V = molar volume
φ = volume fraction
C = $\sqrt{\frac{U}{V}}$ = solubility parameter
U = ΔH_{evap} = cohesion energy $\approx$ specific heat of evaporation
M = molecular weight
ϱ = density
X = mole fraction

Subscripts

m = moving phase
M = solute
Mixt. = mixture
s = stationary phase
S = solvent

$$a = \frac{A_m}{A_s}\left(\frac{1}{R_F} - 1\right) \tag{1}$$

Since the free energy of transfer of one mole of the solute is given by:

$$-\Delta\mu = RT \ln a \tag{2}$$

we obtain by substituting (1) in (2):

$$-\Delta\mu = RT \ln \frac{A_m}{A_s}\left(\frac{1}{R_F} - 1\right) \tag{3}$$

MARTIN postulated that $\Delta\mu$ is the result of the addition of the contributions of the various groups in the molecule of the solute, and obtained the following expression for homologous series, with x as repeating group n times:

$$-\frac{\Delta\mu_x}{RT} = \ln \frac{\left(\frac{1}{R_{F_{n+1}}} - 1\right)}{\left(\frac{1}{R_{F_n}} - 1\right)} \tag{4}$$

The validity of the above expression was confirmed by several authors[2–7].

We wish to present a thermodynamical basis for MARTIN'S postulate. According to KETELAAR[8] the solubility of a solute in a regular solution can be represented by:

$$\ln X_M = -\frac{\Delta\mu}{R}\left(\frac{1}{T} - \frac{1}{T'}\right) - \frac{V_M \varphi_s^2}{RT}(C_S - C_M)^2 \tag{5}$$

If we apply this equation to the distribution of a given material between two phases, we have in the case of dilute solutions, where $\varphi \approx 1$:

$$-\Delta\mu = RT \ln \frac{x_{Ms}}{x_{Mm}} = V_M\left[(C_m - C_M)^2 - (C_s - C_M)^2\right] + RT \ln \frac{M_m \varrho_s}{M_s \varrho_m} =$$

$$= V_M\left[C_m^2 + 2\,C_M(C_s - C_m) - C_s^2\right] + RT \ln \frac{M_m \varrho_s}{M_s \varrho_m} \tag{6}$$

The second part of the right side of the above equation changes the expression of $\Delta\mu$ from mole fraction to concentration.

For homologous series this is approximately equal to:

$$-\Delta\mu_x = A + KV_{xm} \tag{7}$$

which is a mathematical expression of MARTIN'S postulate, as mentioned above.

It is interesting to note that COHN AND EDSALL arrived at the same equation on an empirical basis[9]. Moreover, MASON AND WEGIENKA[10] found an empirical relationship between R_F and molar refraction, which is of course an expression for the molar volume.

Let us consider an application of the above equation.

Table I shows the C_S values of most common solvents. In the case of a mixture we use:

$$C_{S\,\text{Mixt.}} = X_{SA}C_A + X_{SB}C_B \ldots . \tag{8}$$

C_M can be calculated according to the structure by using Table II, *i.e.* by dividing the sum of the respective cohesion constants by the calculated molar volume and taking the square root.

TABLE I

THE SOLUBILITY PARAMETER OF VARIOUS SOLVENTS[8]

Solvent	*Solubility parameter*
n-Pentane	7.05
n-Hexane	7.3
Ether	7.4
Carbon tetrachloride	8.6
Cyclohexanone	8.2
Benzene	9.15
Chloroform	9.3
Chlorobenzene	9.5
Dioxane	9.7
Carbon disulphide	10.0
Pyridine	10.7
Nitrobenzene	11.6
Ethanol	12.7
Methanol	12.7
Water	23.4

TABLE II

THE COHESION ENERGY VOLUME OF VARIOUS CHEMICAL GROUPS*

Group	*Cohesion energy cal. per mole*	*Volume c.c. per mole*
CH_3	1,780	20.1
$=CH_2$	1,780	22.3
$-CH_2-$	890	13.8
$=CH-$ aliphatic	990	7.3
$=CH-$ aromatic	990	11.0
$\equiv CH$	— 380	7.2
–O–	1,630	10.5
–OH	7,250	17.0
=CO	4,270	12.9
–CHO	4,700	19.3
–COOH**	8,970	30.4
–COOMe aliphatic	5,600	60.0
–COOMe aromatic	5,600	44.1
–COOEt aliphatic	6,230	77.0
–COOEt aromatic	6,230	57.8
$-NH_2$***	3,530	14.5
–Cl	3,400	22.0
–F	1,200	7.0
–Br	4,300	28.0
–I	5,040	32.0
$-NO_2$	7,200	13.0
–SH	4,250	32.0
$-CONH_2$	13,200	27.4
–CONH–	10,60	21.0
=	—	8.0
–H	—	6.5
$\left[\begin{array}{l}NH_2\\H_2C\text{–}COOH\end{array}\right.$ as zwitterion	13,390	45.4

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* The data in this Table are based on refs. [11-14].

** For ionization of carboxyl group deduct 10.0 c.c.

*** For ionization of amino group deduct 17.0 c.c.

Varying contractions for hydrogen bonds and ring systems.

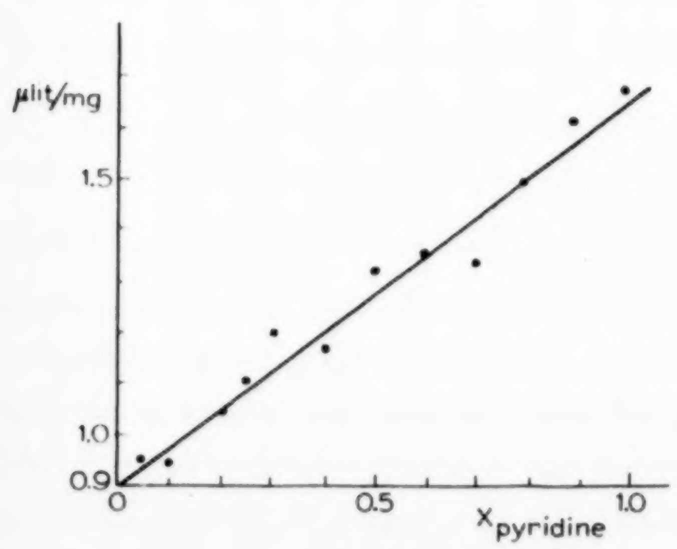
TABLE III

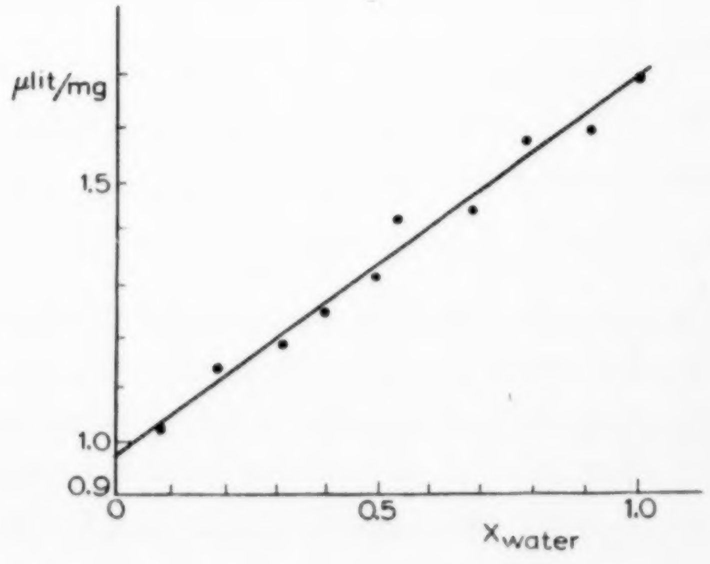
THE VOLUME OF MOVING PHASE FOR VARIOUS SOLVENTS
Whatman No. 1 filter paper at room temperature (≈ 25°).

Solvent	*Volume, μl/mg weight of filter paper*
Ligroin	0.98
Chloroform	0.93
Carbon tetrachloride	0.92
Benzene	0.90
Toluene	0.90
Dioxane	0.98
Butanol	0.97
Amyl alcohol	0.92
Ethyl acetate	0.99
Ethanol 96%	1.10
Acetone	1.10
Acetic acid	1.21
Methanol	1.25
Pyridine	1.60
Water	1.66

Moreover, we can assume that the stationary phase always has the same composition, *i.e.* pure water, independent of the composition of the moving phase. This should be true at least for solvents that cause no swelling of the filter paper. The above assumption, together with the calculated constants, allows the calculation of $\Delta\mu$.

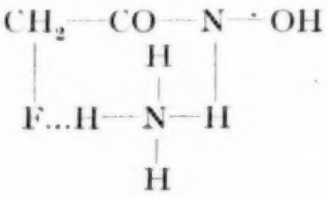
In order to calculate R_F it is necessary to have information about the value of $\frac{\Lambda_m}{\Lambda_s}$. We found experimentally that it is fairly constant for solvents that do not swell the cellulose (Table III). When the moving phase is composed of a mixture of two solvents, one causing and the other not causing swelling, $\frac{\Lambda_m}{\Lambda_s}$ wil be a function of the mole fraction of the solvent that causes the swelling (Figs. 1 and 2). Since the filter paper used in our experiments contained 30% water, and since the data in Table III and Figs. 1 and 2 are given per mg filter paper, the results should be multiplied by 3.3 in order to obtain $\frac{\Lambda_m}{\Lambda_s}$.





We must mention that there is a serious drawback in the actual calculations. When calculating the value in the square brackets in eqn. 6, a small difference between two large numbers is obtained. An error here can cause a difference of 100% or more in the value of R_F, as a result of the inaccuracy of the constants used. By using more accurate constants, the calculated R_F should be closer to the actual value. Nevertheless we can use the above theory for choosing the appropriate solvent for a given material, or for differentiating between two materials. Moreover, the thermodynamical derivation gives some insight into the structures of related substances through their R_F values and can explain relative R_F values.

It is well known[15] that the separation of materials differing only by the replacement of a CH_3 group by a CH_2F group, is extremely difficult. Table II also shows that, in view of the identical molar volumes and the small difference in cohesion constants, the calculated R_F should be similar, the more so as the other groups in the molecule further reduce possible differences. Since we know that hydrogen bonding and cyclization reduces the molar volume, we should expect different R_F values for the ammonium salt of $FCH_2CONHOH$ as compared to $CH_3CONHOH$. This was shown to the case by BERGMANN AND SEGAL[15], and was related to the structure:



The molecular volume of acid hydroxamates increase in the order:

$$FCH_2- < CH_3- < CH_3CH_2- < ClCH_2- < CH_3CH_2CH_2- < BrCH_2-$$

On the other hand the above authors found that the order of the R_F values is:

$$BrCH_2- \approx ClCH_2- < FCH_2- < CH_3- < CH_3CH_2- < CH_3CH_2CH_2-$$

This obvious discrepancy can be clarified as follows:

In eqn. (6) we have two variables, which are dependent on the molecular structure of the moving material, namely: the molar volume (V_M) and the solubility parameter (C_M). In homologous series according to the relative importance of the changes, either factor can dominate and determine the change in the R_F value.

In the above example the equal R_F values for $ClCH_2CONHOH$ and $BrCH_2CONHOH$ can be explained by the fact that according to Table II the cohesion energy of the $ClCH_2$ group $\left(\frac{4390}{35.8} = 122\right)$ is almost equal to that of CH_2Br $\left(\frac{5290}{41.8} = 126\right)$. On substituting the hydrogen by a halogen the cohesion energy increases since the value for a CH_3 group is only $\frac{1780}{20.1} = 89$. According to eqn. (6), the increase of C_M lowers the absolute value in the square brackets, since in our case $C_s > C_m$. This increases the difference between the two terms of eqn. (6), increasing $-\Delta\mu$, which causes a lowering of R_F according to eqn. (3). Internal hydrogen bonding decreases V_m causing the same effect. On the other hand in homologous series C_s is approximately constant; only V_M increases which causes the R_F to increase too.

Finally the following consideration makes it possible to measure the interfering effects. When chromatography is carried out with distilled water, eqn. (6) will give $-\Delta\mu = 0$ and from eqn. (3) we obtain

$$\frac{1}{R_F} = \frac{A_s}{A_m} + 1 \tag{9}$$

i.e. R_F is independent of molecular volume and constant (≈ 0.88), which was found experimentally to be true for such widely differing compounds as: xanthine, coffeine, uric acid, tetramethyluric acid, urea, glycine, leucine, acetohydroxamic acid. We postulate that any deviation from this limit is a measure of the effect of adsorption, precipitation, etc.

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Chromatography on paper impregnated with ion exchange resins
IV. The adsorption of metal ions on Dowex-50 from dilute HF solution

In this note we wish to describe a simple method for determining whether a metal ion in a given solution adsorbs on a cation exchanger. For this purpose KRAUS[1] recommends running short column chromatograms. Ion exchange papers yield the same information and offer perhaps some advantages over both columns and equilibrium studies, since the bands can be detected on the paper after development by the use of suitable spraying reagents. In the case of HF solutions it is also of advantage that only a minimum of equipment is required.

The distribution of cations between ether and aqueous HF solutions was studied by BOCK AND HERRMANN[2] and partition chromatographic studies with solvents and HF or solvents–HF–mineral acids were carried out in the author's laboratory by KERTES[3], VERNOIS[4] and others[5]. Also CROUTHAMEL AND FUDGE[6] described some paper chromatographic studies with fission products. Anion exchange properties of numerous metal ions in HF were recorded by KRAUS and his coworkers[7].

We shall report here the affinity of a number of metal ions in dilute HF to Dowex-50 as this was considered of interest for possible separations of protactinium from other elements. Dowex-50 paper was prepared as already described[8] and developed in polythene containers by ascending development. As solvent a 2% v/v solutions of conc. HF (containing 40% HF) in water was used. The solutions of the metal ions were prepared by dissolving a salt which does not contain a complexing anion (usually the nitrate) or an oxide in 2% HF in small polythene beakers. Since in the cases where the metal ion is adsorbed, the anion moves near the liquid front, the anion does not interfere as would be the case in equilibrium studies.

The following metal ions were found to stay at the point of origin or move only a short distance (below R_F 0.1): Tl, Ag, Cd, Mn, Ni, Co, Cu and Zn. Numerous metal ions moved at or little below the liquid front: Fe(III), U(VI), Zr(IV), Pa(V) (as ^{233}Pa tracer), Ta(V), Nb(V), Ti(IV), Be(II), Al(III), Mo(VI) and Cr(VI). Tetravalent vanadium (dissolved in HF as $V(SO_4)_2$) moves with an R_F 0.35. Metal ions such as alkaline earths and rare earths were not studied as they are rather insoluble in dilute HF.

Dowex-50 or Dowex-50 papers may thus be employed for separating a number of divalent transition elements from Pa(V) or any other of the fast-moving metal ions. Zn and Cd which complex readily in HCl are strongly adsorbed from HF solution.

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Paper-chromatographic identification of organic acids resulting from fermentations

Organic acids, as primary and secondary products of fermentations, were investigated by paper chromatography. The application of this technique to the study of fermentations is vast and very important. Industrial fermentations are generally controlled by titration of the acid. In this way the total acid content is determined, without any indication as to which acids are responsible for the acidity. In research work paper chromatography can contribute much to the study of the biochemistry of each process, since it enables the order to be determined in which the acids are formed during fermentation.

Materials and methods

Ascending chromatography[1] on Whatman No. 1 paper was the method employed. The solvent that gave best results was propyl alcohol–ammonia (70:30 v/v) (ISHERWOOD[2]), development being carried out for 7 h at room temperature. For the detection of the spots bromocresol green solution was used according to KENNEDY[3], except that water was replaced by alcohol, and spraying was followed by 10 min heating at 80°.

The identification was made by comparison of the R_F values with standard 1% solutions of organic acids and, in some cases, by specific reactions. Volatile acids were used in the form of their ammonium salts[3]; because of the instability of these salts the preparation, development and detection of the chromatogram should be made on the same day.

The fermentations were carried out following the techniques and conditions described by PRESCOTT AND DUNN[4].

Results

Alcoholic, lactic, acetic and citric fermentations were tested in this investigation. Each mash was chromatographed before and after fermentation, together with the standard organic acids (Figs. 1 and 2).

The raw materials, conditions and results of each process are set forth in Table I.

Discussion

Alcoholic fermentation. Succinic and acetic acids were identified as by-products of the process. Regardless of the quality of the presence of raw material, the presence of succinic acid was observed also in some initial mashes of molasses, which could certainly be ascribed to a beginning of auto-fermentation.

Lactic fermentation. When skim milk was used as raw material both lactic and

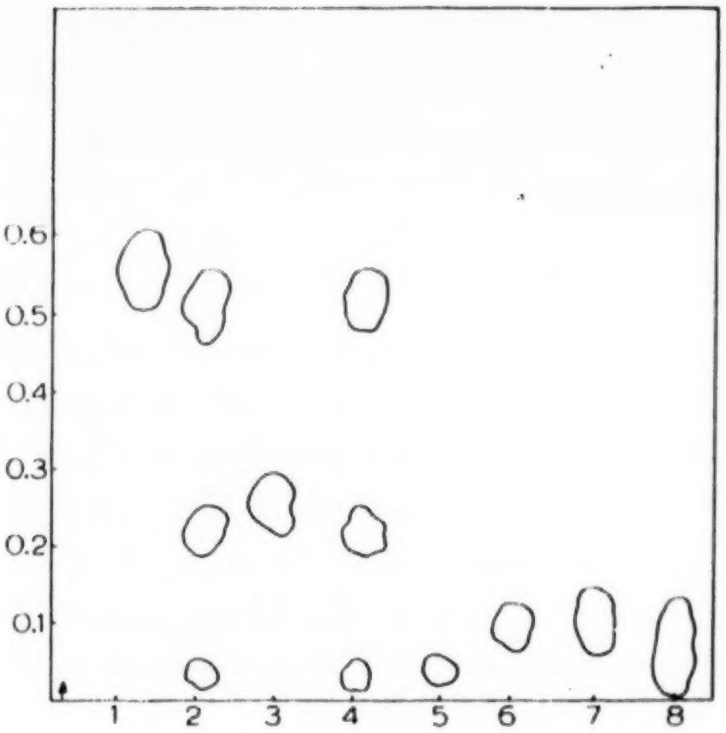

Fig. 1

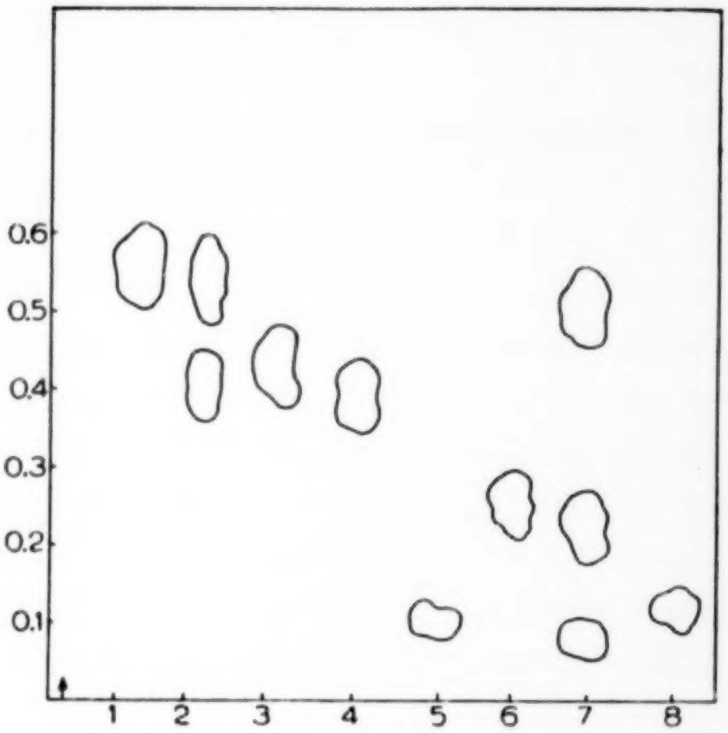

Fig. 2

Fig. 1. (1) acetic acid; (2) alcoholic fermentation (molasses); (3) succinic acid; (4) alcoholic fermentation (glucose); (5) molasses mash; (6) citric acid; (7) citric fermentation; (8) oxalic acid.

Fig. 2. (1) acetic acid; (2) lactic fermentation (skim milk); (3) lactic acid; (4) lactic fermentation (molasses); (5) tartaric acid; (6) succinic acid; (7) acetic fermentation; (8) malic acid.

acetic acids were identified. When molasses mash was used only lactic acid appeared at the end of the fermentation.

Acetic fermentation. Acetic, tartaric and succinic acids were identified and these are therefore responsible for the acidity values found in titrations carried out during fermentation. Since the R_F value of tartaric acid is similar to those of citric, oxalic and malic acids, the detection was carried out with a solution of silver nitrate (ISHERWOOD[2]), which gives a black spot for tartaric acid and white ones for the three others.

TABLE I

ORGANIC ACIDS IDENTIFIED IN FERMENTATIONS

Fermentation	*Raw material*	*Microorganism*	*Conditions*				*Acids identified*
			°Bx	*°C*	*pH*	*time*	
Alcoholic	molasses	*Saccharomyces cerevisiae* ATCC 764	17	25	4.5	48 h	acetic, succinic
Alcoholic	sucrose	*Saccharomyces cerevisiae* ATCC 764	12	25	4.5	48 h	acetic, succinic
Alcoholic	glucose	baker's yeast (Fleischmann)		25	4.5	48 h	succinic
Lactic	skim milk	*Lactobacillus casei* 7460		37	6.5	72 h	acetic, lactic
Lactic	molasses	*Lactobacillus casei* 7460	12	37	6.7	72 h	lactic
Acetic	ethyl alcohol	*Acetobacter suboxydans*		27		10 d	acetic, tartaric, succinic
Citric	sucrose	*Aspergillus niger* 1015		25	3.5	10 d	citric

Citric fermentation. Only citric acid was identified although oxalic acid frequently appears during the process. These two acids, the R_F values of which are very similar, can be distinguished by elution with pyridine and acetic anhydride according to FURT AND HERMANN[5], a red colour being obtained for citric acid and gas evolution for oxalic acid.

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Chromatography of neutral amino acids on columns of cellulose powder

In a previous paper[1] we described the fractionation of peptides in columns of cellulose powder using a volatile solvent, *viz.*, a mixture of ethyl alcohol and water. In spite of the great number of existing methods for the separation of amino acids we thought it would be interesting to apply this system to the fractionation of these acids.

Two sizes of columns were used in this work, smaller ones of 31 × 0.9 cm and larger ones of 100 × 0.9 cm. The columns were prepared by pouring into a glass tube a slurry obtained by suspending 1 part of cellulose powder (Whatman standard grade) in 3 parts of a mixture of ethyl alcohol and water of the appropriate concentration. After the cellulose powder had sedimented a pressure of 15 lbs. per sq. in. was applied. The columns were then washed under pressure with about 2 l of the ethyl alcohol–water mixture. These columns can be used many times, provided they are washed between runs with about 1 l of absolute ethyl alcohol and then equilibrated with the solvent to be used for the chromatography.

The columns were mounted on an automatic fraction collector and the flow was adjusted to 2.5 ml/h; 0.75 ml fractions were collected. Alternate fractions were analysed by a modification of the ninhydrin method of TROLL AND CANNAN[2] and by one-dimensional paper chromatography.

The mixture of amino acids to be separated was dissolved in 1 ml of the appropriate solvent, applied to the top of the columns and washed down with two 1 ml portions of the same solvent.

One of the mixtures used in these separations contained phenylalanine, glycine, leucine, proline, threonine, alanine, methionine, serine and valine. A simple mixture

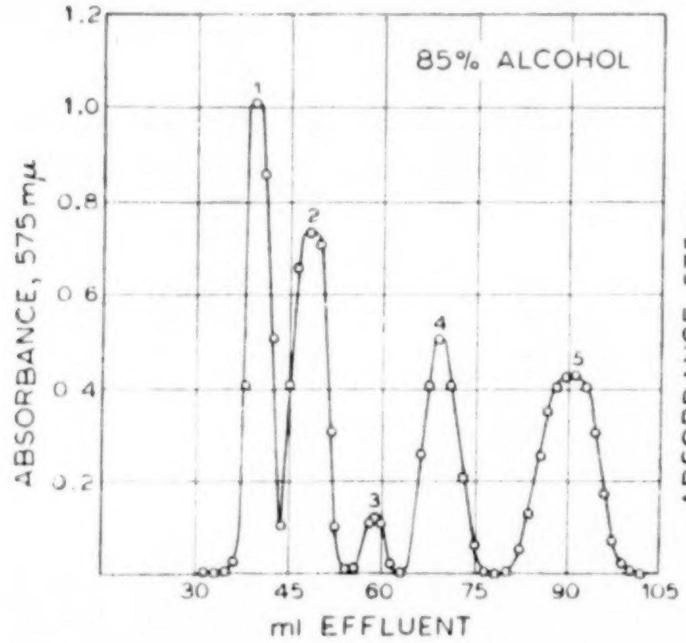

Fig. 1. Chromatographic separation of a mixture containing 1 μmole of each of the following amino acids: DL-leucine (1), DL-phenylalanine (2), L-proline (3), DL-threonine (4) and glycine (5). Eluant: mixture of ethyl alcohol (85%) and water. Column dimensions: 30 × 0.9 cm. Flow rate: 2.5 ml/h. Fractions of 0.75 ml each.

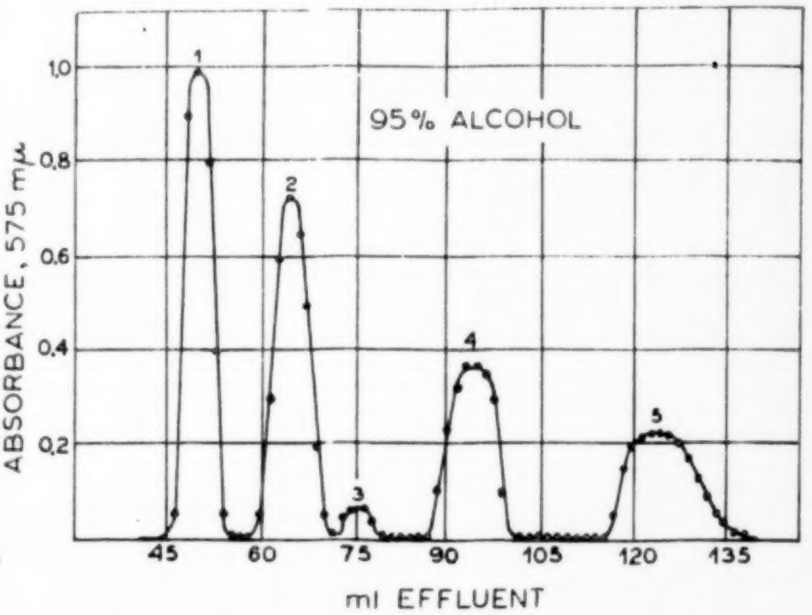

Fig. 2. Chromatographic separation of a mixture containing 1 μmole of each of the following amino acids: DL-leucine (1), DL-phenylalanine (2), L-proline (3), DL-threonine (4) and glycine (5). Eluant: mixture of ethyl alcohol (95%) and water. Column dimensions: 30 × 0.9 cm. Flow rate: 2.5 ml/h. Fractions of 0.75 ml each.

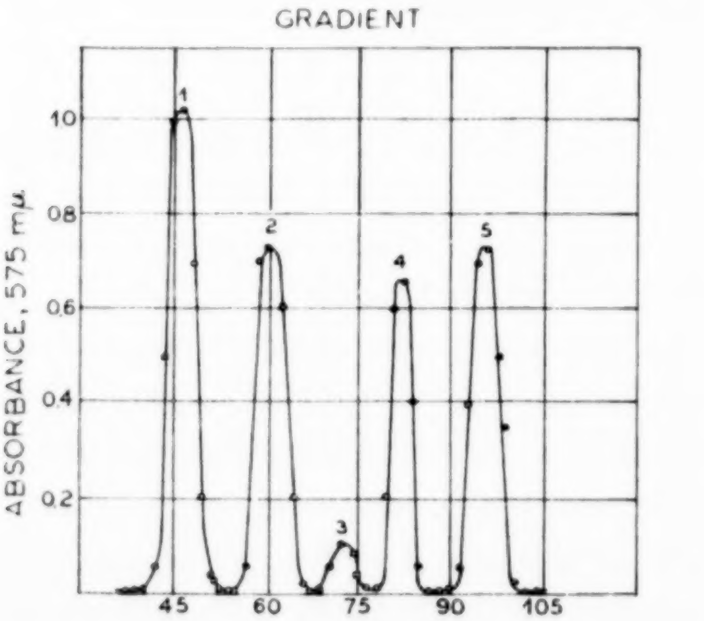

Fig. 3. Chromatographic separation of a mixture containing 1 μmole of each of the following amino acids: DL-leucine (1), DL-phenylalanine (2), L-proline (3), DL-threonine (4) and glycine. Gradient elution. Column dimensions: 30 × 0.9 cm. Flow rate: 2.5 ml/h. Fractions of 0.75 ml each.

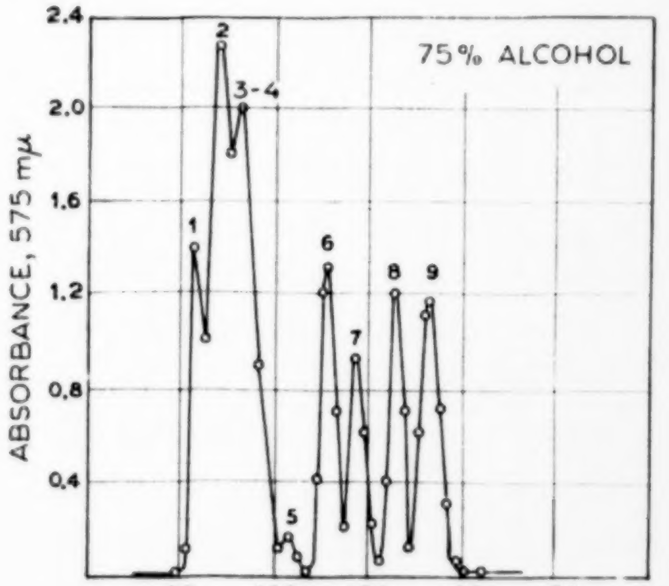

Fig. 4. Chromatographic separation of a mixture containing 0.7 μmole of each of the following amino acids: DL-leucine (1), DL-valine (2), DL-phenylalanine (3), DL-methionine (4), L-proline (5), DL-alanine (6), DL-threonine (7), DL-phenylalanine (3), DL-methionine (4), DL-serine (8) and glycine (9). Eluant: mixture of ethyl alcohol (75%) and water. Column dimensions: 100 × 0.9 cm. Flow rate: 2.5 ml/h. Fractions of 0.75 ml each.

containing only phenylalanine, glycine, leucine, proline and threonine was also used.

When a concentration gradient was used, this was obtained by the automatic addition of a 40% ethyl alcohol to 325 ml of 95% ethyl alcohol.

In Figs. 1 and 2 the fractionation of the mixture containing five amino acids, using 85 and 95% ethyl alcohol, is shown. In the first case all the fractions came out sharply, but the separation of the leucine and phenylalanine peaks is not complete. Using 95% alcohol all the five amino acids are separated with pronounced loss of sharpness for the peaks of threonine and glycine. Complete separation and sharpness of all peaks can, however, be obtained by using gradient elution as shown in Fig. 3.

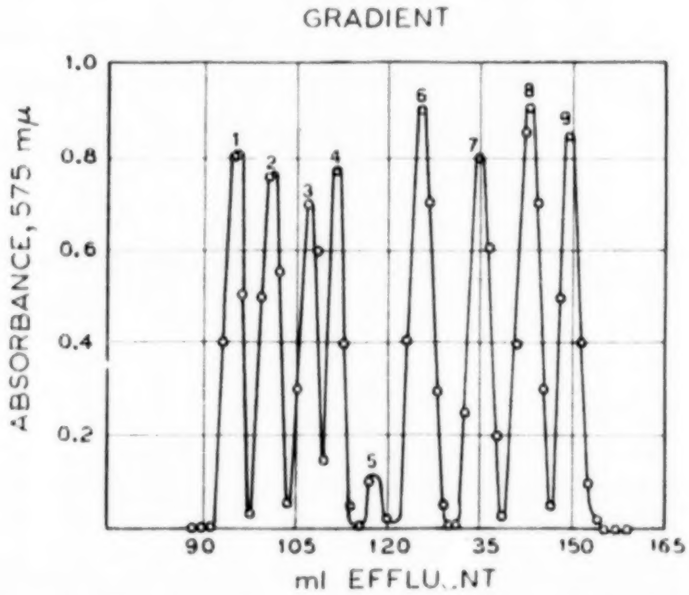

Fig. 5. Chromatographic separation of a mixture containing 0.7 μmole of each of the following amino acids: DL-leucine (1), DL-valine (2), DL-phenylalanine (3), DL-methionine (4), L-proline (5), DL-alanine (6), DL-threonine (7), DL-serine (8) and glycine (9). Gradient elution. Column dimensions: 100 × 0.9 cm. Flow rate: 3 ml/h. Fractions of 0.75 ml each.

With the mixture of nine amino acids good separation could not be achieved by employing a single concentration of alcohol. The best results were observed in this case when the solvent was 75% alcohol (Fig. 4), but this is still much inferior to the separation obtained when gradient elution was used (Fig. 5).

The method presented in this paper may prove valuable for preparative work where the volatility of the solvent is an advantage.

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A note on the interference of cations and anions in paper electrophoresis

The independent movement of cations and anions in the electric field is no doubt one of the fundamentals of the ionic theory and has been repeatedly observed in paper electrophoresis. We wish to report here one apparent deviation from independent migration, which was observed while investigating complexing between cations and anions.

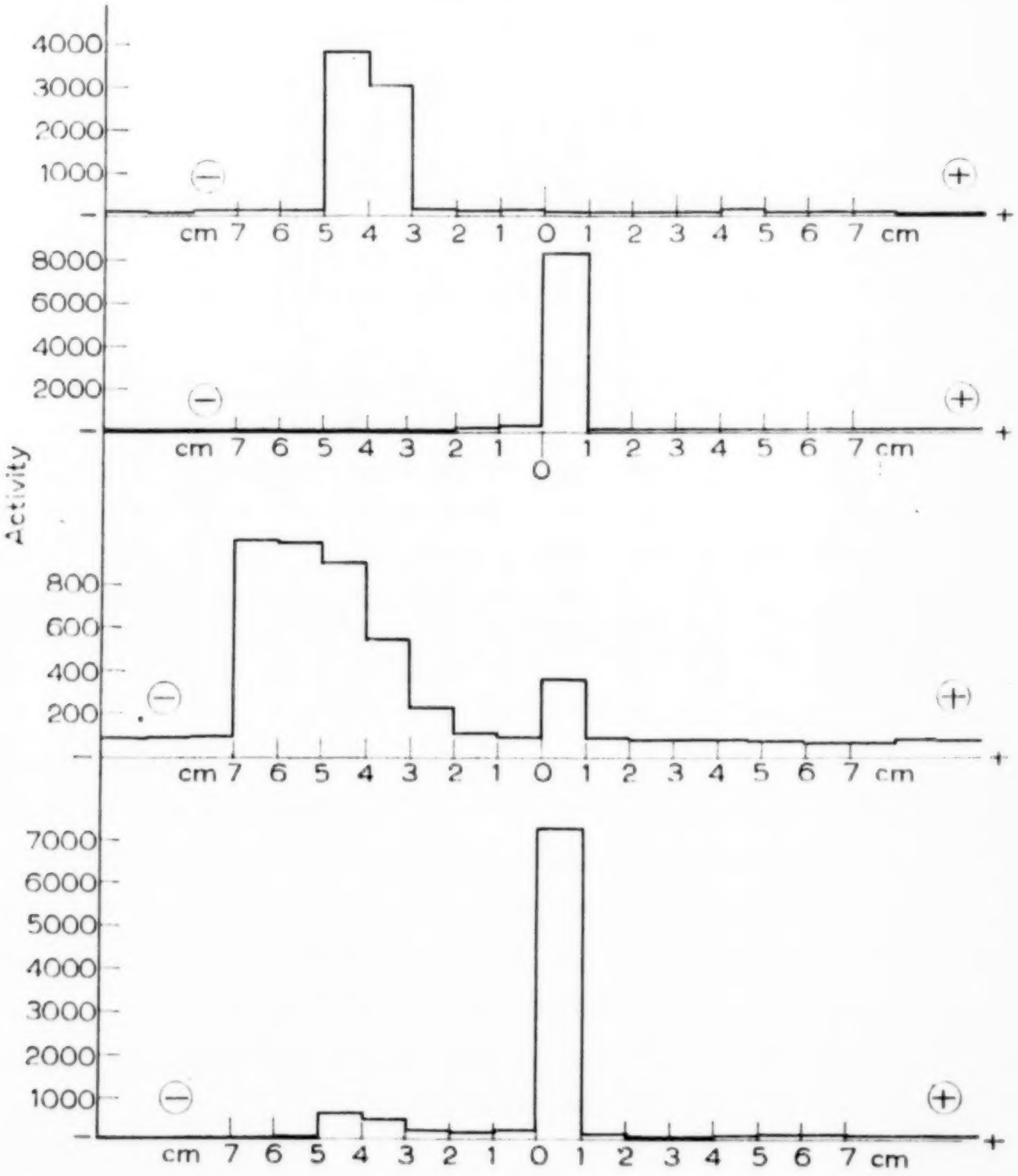

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Fig. 1a. Electropherogram of a solution of ^{137}Cs tracer in $N/2$ HCl as electrolyte with 300 V for 27 min (glass plate technique).

Fig. 1b. Electropherogram of a mixture of $M/10$ $K_4Fe(CN)_6$ with ^{137}Cs tracer. Conditions as in Fig. 1a.

Fig. 1c. Electropherogram of a mixture of ^{137}Cs with inactive CsCl and $M/10$ $K_4Fe(CN)_6$. Conditions as in Fig. 1a.

Fig. 1d. Electropherogram of a mixture of ^{137}Cs tracer with M/10,000 $K_4Fe(CN)_6$. Conditions as in Fig. 1a.

Solutions of caesium chloride and potassium ferrocyanide in water or dilute HCl may be mixed without forming a precipitate. When such mixtures are electrophorized in acid solution on paper, all or part of the Cs (depending on its concentration) does not move from the start. More remarkable still is that tracer quantities of ^{137}Cs will be completely retained at the start when mixed with ferro- or ferricyanide while they move rather quickly when placed on the paper alone.

Fig. 1 shows several typical electropherograms.

We should like to offer the following explanation for this phenomenon: Caesium forms extremely insoluble double ferrocyanides with Zn and other transition metals which have been used to remove tracer Cs quantitatively from solution. Such precipitates seem to form with the traces of Cu or Zn present in the paper as impurity. A neutral soluble particle is not formed, as paper chromatography of ^{137}Cs mixed with ferrocyanide with aqueous solvents gives a spot of R_F 0.

We should like to offer this observation as it might suggest similar explanations for quite a number of "ghost spots" and "comets" encountered in paper electrophoresis.

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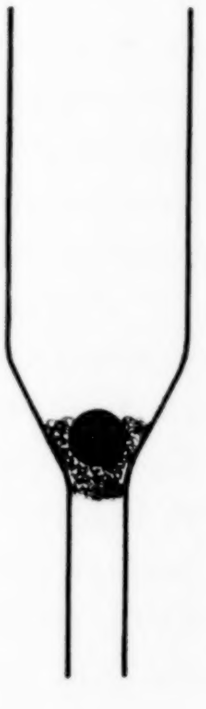
Practical method of closing small columns in column chromatography

In a study on the chlorophylls in lichens it proved to be necessary to reduce the quantity of cotton wool used to retain the adsorbent more than is allowed in the well-known methods[1,2]. A glass bead wrapped up in a thin layer of cotton wool was found to be handy. After the coated glass bead has been dropped into the tube, it is pressed down gently and the cotton wool on the top of the glass bead is "brushed" into the crevice between the tube and the glass bead (see Fig. 1).



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Séparation et microanalyse par chromatographie sur colonne adsorbante d'azote et d'oxyde azotique dans des mélanges d'azote et de ses oxydes

Pour obtenir le rendement quantitatif de divers gaz produits par irradiation gamma de solutions aqueuses d'hydroxylamine[1], nous avons été amenés à rechercher une méthode de séparation pour des microquantités d'azote et d'oxygène, puis d'azote et d'oxyde azotique en présence d'autres dérivés oxydés comme N_2O et N_2O_4. Nous avons pensé utiliser la séparation par chromatographie gazeuse mais les techniques publiées sur ce problème ne permettaient pas des dosages précis et rapides (voir par exemple réf. 2).

Nous avons donc été amenés à mettre au point une méthode originale en utilisant l'appareil Griffin et George.

Conditions expérimentales

(*a*) *Colonne d'adsorption.* Nous avons pris une colonne de 8 mm de diamètre et de 3.2 m de long, remplie à une densité de 0.33 g/cm^3 par du silicate d'alumine et de calcium (tamis moléculaire type 5 A Linde). Cette substance adsorbante a été choisie de préférence au charbon activé et au gel de silice. En effet elle est la seule à permettre la séparation de NO et N_2; elle adsorbe définitivement N_2O. Elle est "activée" par chauffage sous vide à 350°. L'éluant utilisé a été de l'hydrogène et les meilleures conditions de fonctionnement correspondent à un débit de 1.4 l/h, à une température de colonne de 86° et un courant de chauffage de la cellule de détection par thermoconductivité de 175 mA.

(*b*) *Introduction des gaz.* Pour nous permettre d'analyser les gaz produits par irradiation aux rayons gamma, nous avons été amenés à modifier légèrement l'appareil conçu surtout pour introduire en tête de la colonne, à l'aide d'une seringue, des liquides qui sont ensuite vaporisés. Nous avons intercallé entre l'arrivée d'hydrogène et l'entrée de la colonne un récipient de 50 cm^3 fermé par deux robinets à 3 voies permettant le passage de l'hydrogène en dehors ou à travers le volume du récipient. Ce dispositif est fixé sur la colonne par l'intermédiaire de rodages sphériques normalisés. On peut donc prélever des gaz d'un volume irradié quelconque et les refouler dans le récipient précédemment décrit, à l'aide d'une pompe Toepler par exemple; on respecte de la sorte les conditions d'instantanéité et de ponctualité pour l'introduction des gaz à analyser dans la colonne et l'élargissement des pics sur le graphique enregistré est négligeable.

Résultats

Ce dispositif nous a permis d'analyser des mélanges contenant entre 10 et 500 mm^3 d'azote ou d'oxygène avec une erreur maxima de $\pm$ 4 mm^3, ou des mélanges contenant de 10 mm^3 à 500 mm^3 d'azote et 50 à 500 mm^3 de NO à la pression atmosphérique. (erreur maximum, pour NO $\pm$ 20 mm^3 entre 50 et 150 mm^3 et $\pm$ 6 mm^3 entre 150 et

500 mm³). La présence de N_2O ne gêne pas la séparation des autres gaz, bien qu'il soit entièrement adsorbé. On peut le doser par ailleurs après l'avoir piégé dans un récipient refroidi à l'azote liquide.

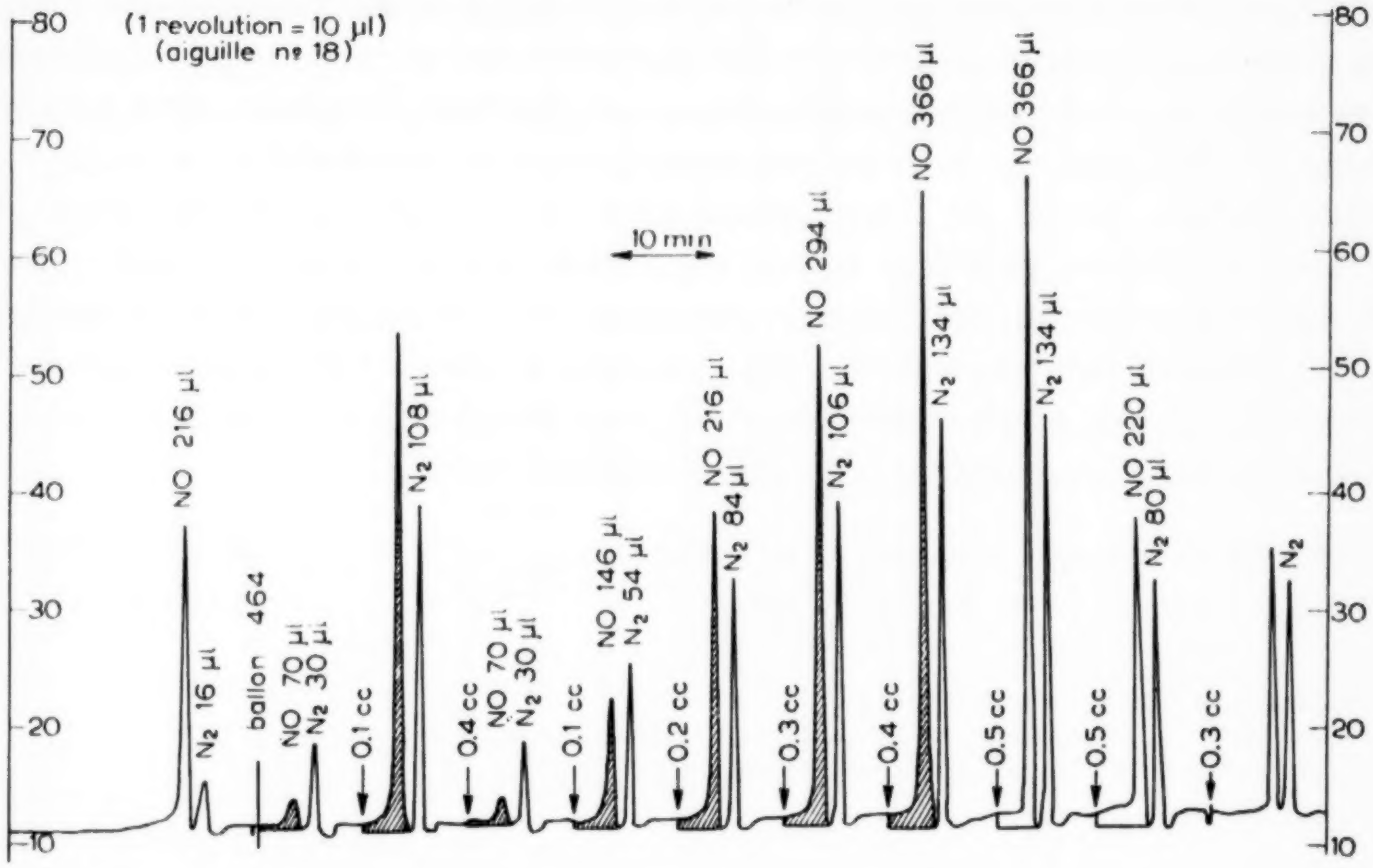

Fig. 1

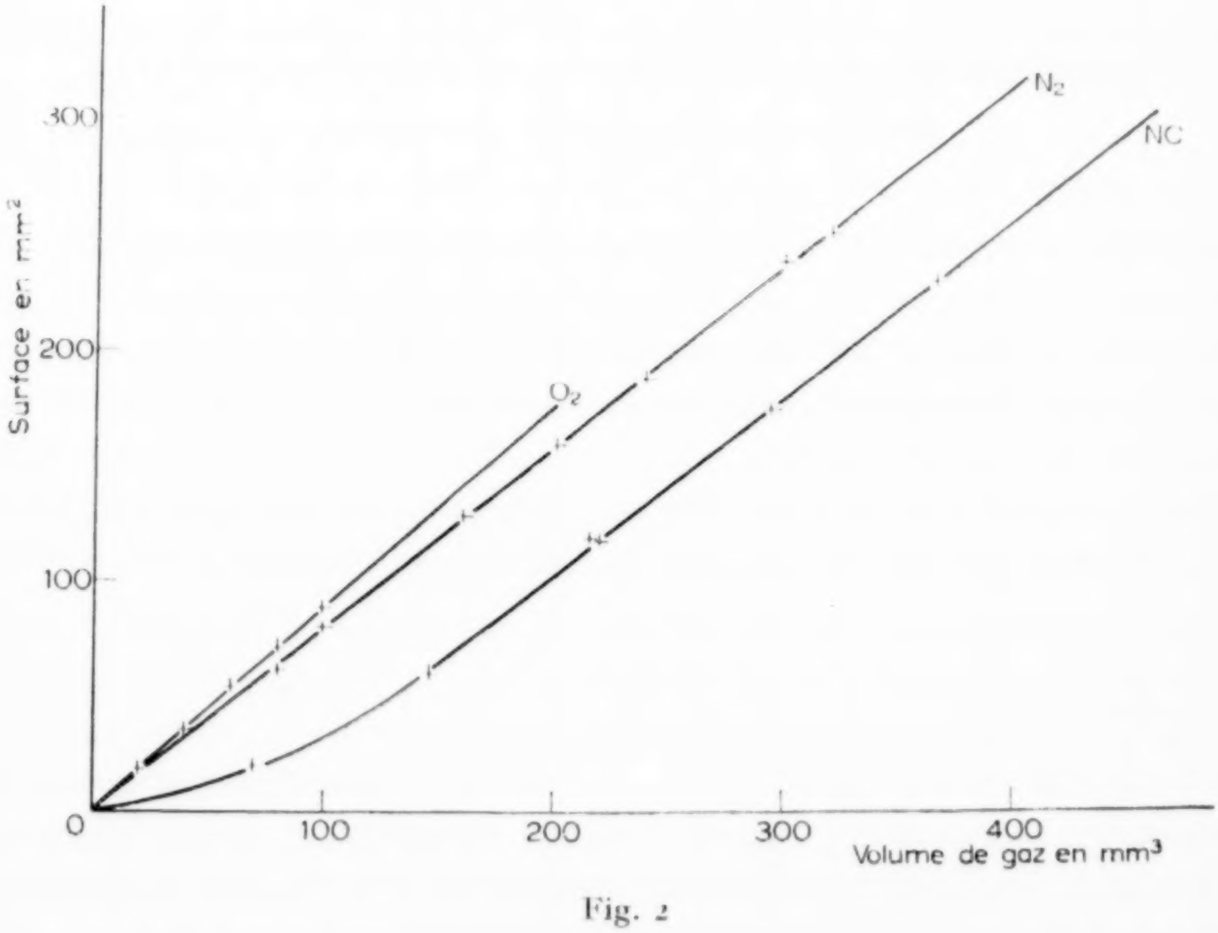

Fig. 2

On peut voir sur le diagramme de la Fig. 1 que pour l'oxygène et l'azote les courbes sont parfaitement assimilables à des courbes de Gauss et la hauteur des pics est proportionnelle à la quantité de gaz contenue dans l'échantillon. Le temps de rétention est de 173 sec pour O_2, 300 sec pour N_2, et 400 sec pour NO. L'analyse quantitative de NO est plus délicate car son élution n'est pas totale. Il reste environ 40 mm^3 sur la colonne adsorbés de façon très durable (plus de deux heures dans nos conditions). Pour avoir des résultats quantitatifs très reproductibles, il faut fournir à la colonne une certaine quantité de NO pour atteindre un pseudo équilibre. On fait donc passer 200 mm^3 de NO quatre fois avant d'utiliser la colonne pour l'analyse désirée. On peut alors opérer dans les deux heures qui suivent et obtenir une reproductibilité parfaite. Le pic est dissymétrique et il convient de mesurer sa surface. La méthode est d'autre part plus précise si on opère sur des quantités supérieures à 150 mm^3. On a donc intérêt à ajouter aux échantillons à analyser de 100 à 200 mm^3 de NO préalablement mesurés. La Fig. 2 montre d'ailleurs l'étalonnage donnant la surface des pics en fonction du volume en mm^3 introduit sur la colonne et mesuré préalablement à la jauge de Mac Leod sous pression réduite.

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Reçu le 5 novembre 1958

Homogenization and extraction of biological material for chromatographic purposes

The preparation of biological samples for chromatographic analysis usually involves homogenization and extraction. These procedures are very laborious and they expose the investigated samples to loss and enzymic changes. Moreover they require at least decigram amounts of the preparation, whereas for the paper chromatographic analysis a hundredth of this amount suffices.

A simple method was elaborated in our laboratory for the homogenization and extraction of small amounts of biological material directly onto the chromatographic paper.

Homogenization

The new homogenization procedure is based on the new method of botanical investigation devised by MEDVEDEV[1,2] and the method for studying simple compounds in plants described by GREENSHIELDS[3], as well as on the method of MORGAN AND WICKSTROM[4]. The chromatographic paper is prepared as for one-dimensional, two-dimensional or circular paper chromatography. The preparation to be investigated (hyphae, a small piece of leaf, corola, thick section of fruit, stems, etc.) is placed on the starting point; plastic foils (6 × 6 cm) are placed under the chromatographic paper

and over the preparation and then the whole is placed between two polished steel plates (Fig. 1) and submitted to transient high pressure by means of a blow with a hammer or a suitable spring apparatus. The transient high pressure causes the plant cells to burst so that their contents flow onto the paper where they are instantly absorbed. The spot is then dried with a hair dryer to interrupt the enzymic processes in the juice. To prevent loss of juice by absorption on the upper plastic foil the sample can be placed between two chromatographic papers. After pressing, the two spots obtained are transferred onto one chromatogram by some method of rechromatography[5,6].

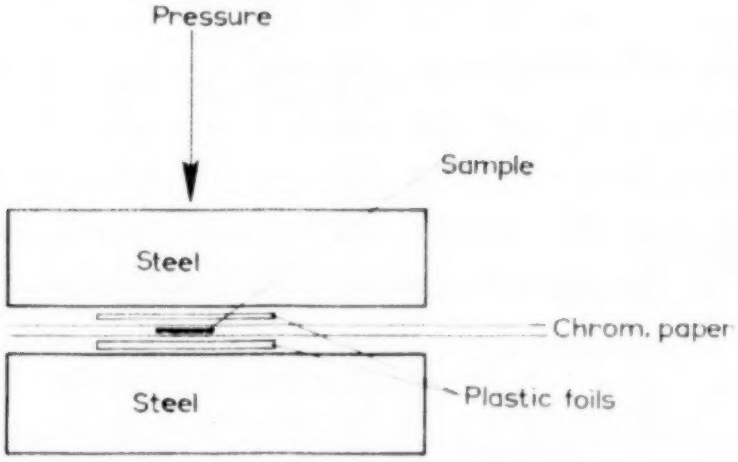

Fig. 1

By the isotopic method using ^{32}P it was confirmed that less than 0.1% of phosphorus contained in plant juice is absorbed on the plastic foil, if the sample is placed between two papers.

In the investigation of virus necrosis on leaves, infiltrated spaces of leaf etc., small pieces of leaf can be cut out with a cork borer or other perforator (1–4 mm in diameter).

It is also possible to apply this method to the investigation of zoological preparations, such as whole small insects, parasites or separate organs (glands, nerves etc.), small sections of muscle, skin, etc.

Extraction

If the total juice pressed out of the preparation cannot be analysed because of contaminating or inhibiting substances, the analysis can be accomplished after extraction of the desired ingredients by a suitable solvent. The extraction can also be carried out on the chromatographic paper.

For this purpose the biological sample is homogenized on the chromatographic paper as described above. When the sample has been subjected to pressure at the starting point, the ingredient of the sample can be extracted by allowing a suitable solvent, instead of the developer, to flow along the chromatogram.

The extracted substances can be collected in a tube according to the method of DENT[7] or on a small tongue of chromatographic paper according to the method of

Oertel[8]. The small tongue of paper with the collected substances can be used as the spot of origin for the new chromatogram[5], or the substances can be extracted with a suitable microextractor for further analysis[9].

If the substance to be investigated is present in dilute solution, it can be concentrated by the method devised by Krzeczkowska[10]. This method can be combined with that of Gregory[6] for transferring a spot from one chromatogram to the origin of another. The combination of these two methods is illustrated in Fig. 2.

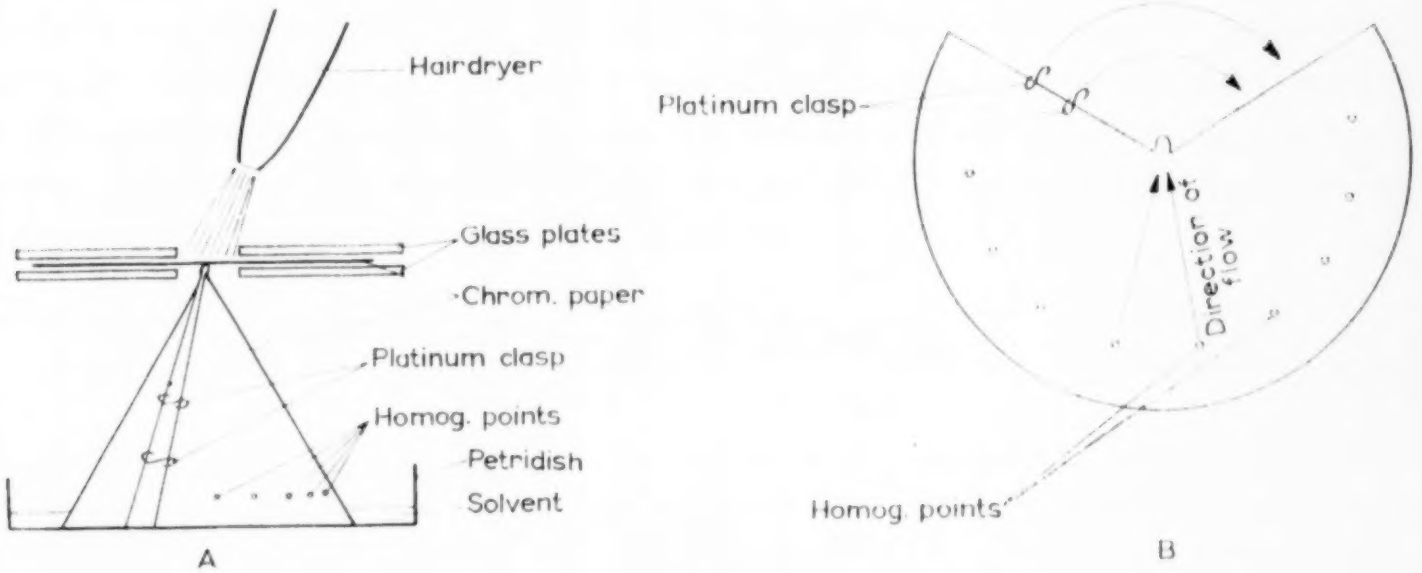

Fig. 2

The same preparation can be extracted several times by using different specific solvents for the various compounds[11].

The method described is especially convenient for radiochromatographic analysis of biological material, because it offers to the possibility of analysing small amounts of the sample and because of the simplicity of the technique.

Acknowledgements

The author wishes to express his thanks to Professor Dr. W. Moycho for his helpful criticism of this work and to Mrs. E. Antoszewska for her kindness in supplying the necessary equipment.

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BOOK REVIEWS

Handbuch der Papierchromatographie, herausgegeben von I. M. HAIS UND K. MACEK; Übersetzung nach der zweiten, überarbeiteten und erweiterten tschechischen Auflage. Band I: *Grundlagen und Technik*, unter Mitarbeit von 20 Fachwissenschaftlern, Gustav Fischer Verlag, Jena, 1958, mit 242 zum Teil farbigen Abbildungen, XXIV, 860 Seiten, Ganzleinen DM 58.40.

Das von HAIS UND MACEK herausgegebene Handbuch der Papierchromatographie, 1. Teil, unterscheidet sich schon rein äusserlich von den anderen Büchern über Papierchromatographie: es ist wohl das umfangreichste und gewichtigste Werk, das bisher über dieses Thema geschrieben wurde. Die einzelnen Beiträge stammen von den Herausgebern und nicht weniger als 20 wissenschaftlichen Mitarbeitern. Dadurch war es möglich, die einzelnen Abschnitte und Anwendungsgebiete von speziell auf dem betreffenden Gebiet arbeitenden Fachleuten schreiben zu lassen. Insgesamt wurden etwa 10,000 Literaturstellen berücksichtigt, die in Form einer Bibliographie den 2. Teil des Handbuches bilden sollen. Im vorliegenden Werk wurde jedoch eine Auswahl getroffen und etwa ein Viertel der genannten Literaturstellen tatsächlich verwendet.

In einem allgemeinen etwa 200 Seiten umfassenden Teil werden Theorie und Grundlagen der Papierchromatographie behandelt und die verschiedenen Arbeitsmethoden beschrieben. Im zweiten etwa 550 Seiten umfassenden Buchabschnitt findet man Untersuchungsmethoden für alle wichtigen organischen und anorganischen Substanzklassen. Ein letzter 65 Seiten langer Teil bringt Rezepte und genaue experimentelle Vorschriften. Das ausführliche Autoren- und Sachregister beschliesst das Werk.

Die Art der Darstellung weicht oft von der bisher üblichen ab. So werden die R_F-Werte weniger in Tabellen, als in sehr übersichtlichen graphischen Darstellungen und Landkarten angegeben. Bei den meisten Stoffklassen wird auch ausführlich auf Regelmässigkeiten und Zusammenhänge zwischen Konstitution bzw. Konstellation und den R_F Werten eingegangen; diese Abschnitte sind vielfach nicht als Referate sondern als Orginalarbeiten der Autoren zu betrachten. Diese beschränken sich auch nicht nur auf die Schilderung der eigentlichen papierchromatographischen Untersuchung, sondern geben auch ausführliche Hinweise auf die Aufbereitung des Untersuchungsmaterials, auf eventuelle Vorreinigungen, Trenn- und Abbaumethoden. Man findet so z.B. ausführliche Kapitel über die Konstitutionsermittlung von Polysacchariden und Polypeptiden.

Für eine spätere Auflage wäre vielleicht die Ausmerzung verschiedener im Deutschen nicht sofort verständlicher Anglizismen, z.B. Wechselwirkung statt Interaktion, anzuregen. Es liessen sich vielleicht auch mehr Querverbindungen zwischen den einzelnen Kapiteln, die ja von verschiedenen Autoren geschrieben sind, durchführen. Es ist auch schade, dass durch die bei der Herausgabe von Handbüchern

unvermeidlichen Verzögerungen, die Literatur nur bis 1956 berücksichtigt werden konnte; es ist aber erstaunlich, dass viele Autoren die weitere Entwicklung richtig vorhersahen.

Das vorliegende Werk stellt eine wesentliche Bereicherung der auf diesem Gebiet vorhandenen Literatur dar.

H. MICHL (Wien)

Advances in Clinical Chemistry, Vol. 1, edited by H. SOBOTKA AND C. P. STEWART. Academic Press Inc., New York, 1958, 398 pp., price $ 10.

Contents

Plasma Iron, by W. N. M. RAMSAY
The Assessment of the Tubular Function of the Kidneys, by B. JOSEPHSON AND J. EK
Protein-Bound Iodine, by A. L. CHANEY
Blood Plasma Levels of Radioactive Iodine-131 in the Diagnosis of Hyperthyroidism, by S. SILVER
Determination of Individual Adrenocortical Steroids, by R. NEHER
The 5-Hydroxyindoles, by C. E. DALGLIESH
Paper Electrophoresis of Proteins and Protein-Bound Substances in Clinical Investigations, by J. A. OWEN
Composition of Body Fluids in Childhood, by B. JOSEPHSON
The Clinical Significance of Alterations in Transaminase Activities of Serum and Other Body Fluids, by F. WROBLEWSKI.

In most chapters chromatographic methods are discussed in relation to the topics dealt with, especially in those by NEHER and DALGLIESH. The reviewer will confine himself, however, to the chapter by OWEN (Melbourne) on the clinical application of paper electrophoresis. From a critical evaluation of about 400 papers OWEN concludes that "the diagnostic value of paper electrophoresis of serum or urinary proteins is severely limited by the occurrence of similar changes in a number of diseases", also "A normal electrophoretic pattern... does not exclude the presence of disease". These are conclusions with which the reviewer heartily agrees.

They seem, however, in some contradiction with a remark concerning the use of visual examination of the electropherograms:"... that visual inspection is subject to considerable error and therefore unsuitable for precise work or for most serial studies". For there appears no need for a greater precision in the quantitative evaluation than that of the biological variation. A large number of results for numerous diseases is well discussed, also such topics as lipids and carbohydrates and the abnormal haemoglobins etc... The review should be very valuable for clinical workers. Some illustrations of electropherograms could have increased its value as a handbook.

M. LEDERER (Paris)

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REVUE D'ENSEMBLE

CHROMATOGRAPHIE SUR PAPIER DES DINITROPHÉNYLAMINOACIDES

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A. INTRODUCTION

Les dinitrophénylaminoacides (DNP-aminoacides) sont des dérivés fréquemment employés dans la chimie des protéines. En effet, le 1-fluoro-2,4-dinitrobenzène (FDNB) se fixe facilement à la température du laboratoire et en milieu légèrement alcalin sur les groupes α-aminés libres d'un acide aminé, d'un peptide ou d'une protéine.

---

* Avec la collaboration technique de Mlle M. T. PICQUÉ

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Bibliographie p. 269/271.

Au cours de cette condensation, le radical dinitrophényle peut également se fixer sur les groupements thiol de la cystéine et phénol de la tyrosine, les groupes ω-aminés des acides diaminés, ainsi que sur le noyau imidazole de l'histidine. L'arginine, les amides des acides dicarboxyliques donnent un dérivé monodinitrophénylé sur leur groupe α-aminé.

De très nombreux dinitrophénylaminoacides (DNP-aminoacides) sont *éthérosolubles*. Cependant quelques uns, comme l'α-mono-DNP-arginine, l'acide DNP-cystéique, l'α-DNP-histidine, l'ε-mono-DNP-lysine, l'O-DNP-tyrosine, la S-DNP-cystéine, l'imidazole-DNP-histidine, sont *hydrosolubles*.

La détermination de l'acide aminé porteur d'un groupe α-aminé libre et en position terminale dans une chaîne peptidique s'effectue très souvent par la méthode des dinitrophénylaminoacides de SANGER.

D'autre part, les acides aminés d'un hydrolysat total ou d'un hydrolysat enzymatique (action de la carboxypeptidase ou de la leucine-aminopeptidase) de protéine peuvent être identifiés et dosés sous la forme de leurs dérivés dinitrophénylés.

L'identification et le dosage des DNP-aminoacides constituent donc des problèmes analytiques primordiaux. Les méthodes de séparation sont principalement chromatographiques.

De nombreuses techniques de chromatographie sur colonnes ont été décrites: chromatographie sur gel de silice (SANGER[76], PORTER ET SANGER[67]); sur gel de silice tamponné (BLACKBURN[12], MIDDLEBROOK[57]); sur Kieselguhr (MILLS[58], BRAUNITZER ET REUTHER[16]); sur Hyflo-Super-Cel tamponné (BELL *et al.*[7]); sur acide silicique, Celite (GREEN ET KAY[31]). Mais il est beaucoup plus commode de se servir de la chromatographie de partage sur papier. D'assez nombreux systèmes solvants de valeur inégale ont été proposés. Actuellement, les séparations sont facilement obtenues à l'aide de plusieurs types de chromatographie bidimensionnelle dont nous décrirons plus loin les modalités. Des méthodes de chromatographie en phase inversée ont également été publiées: sur caoutchouc chloré (PARTRIDGE ET SWAIN[63]), sur papier acétylé (BURTON[18]).

B. PRÉPARATION DES DINITROPHÉNYLAMINOACIDES

Les préparations des DNP-aminoacides de référence ont été décrites dans les articles de SANGER[76], PORTER ET SANGER[67], LEVY ET CHUNG[45], FRAENKEL-CONRAT, HARRIS ET LEVY[28], et RAO ET SOBER[69].

La préparation et les caractéristiques physiques de très nombreux DNP-aminoacides éthérosolubles sont rassemblés dans les articles de RAO ET SOBER[69] et de FRAENKEL-CONRAT, HARRIS ET LEVY[28].

a. Procédés généraux de synthèse

Deux procédés commodes de synthèse sont à retenir.

1. *Procédé de* LEVY ET CHUNG[45]

La condensation se fait en milieu aqueux et à 40°. L'aminoacide (10 mmoles) et le carbonate de sodium anhydre (2 g) sont dissous dans 40 ml d'eau à 40°. Le fluorodinitrobenzène (10 mmoles) est ajouté et le mélange est agité vigoureusement, la température étant maintenue à 40°. Les gouttelettes de fluorodinitrobenzène en suspension disparaissent au bout d'une demi-heure environ, ce qui marque la fin de la réaction. L'acidification (acide chlorhydrique concentré 3 ml) de la solution orange permet la précipitation du DNP-aminoacide, dont la cristallisation est amorcée par frottement. La recristallisation du DNP-aminoacide se fait à partir de mélanges solvants spéciaux (voir Tableau I).

TABLEAU I

SOLVANTS POUR LA PURIFICATION ET LA CRISTALLISATION* DES DNP-AMINOACIDES
(D'après RAO ET SOBER[69])

Éther–éther de pétrole (E: 30–70°)	L-Alanine, acide DL- et L-α-aminobutyrique, DL- et L-valine, acide DL- et L-glutamique, L-norvaline, L-isovaline, DL- et L-leucine, DL-, D- et L-*allo*isoleucine, DL- et L-thréonine, DL- et L-*allo*thréonine, DL-méthionine, DL-éthionine, L-cystine, S-benzyl-L-cystéine, L-phénylalanine, DL- et L-proline, hydroxy-L-proline, acide DL-pipécolique
Méthanol aqueux	Glycocolle, DL- et L-sérine, L-asparagine, L-glutamine
Acétone aqueuse	Acide γ-hydroxy-L-α-aminobutyrique, acide ε-hydroxy-L-α-aminocaproïque, acide L-α,γ-diaminobutyrique, L-arginine, di-DNP-histidine
Acétone–éther	O,N-Di-DNP-L-tyrosine, L-tryptophanne, di-DNP-L-lysine
Acétone–éther de pétrole	Di-DNP-L-ornithine
Éthanol aqueux	β-Alanine

* En général, les DNP-DL-aminoacides cristallisent plus facilement que les dérivés correspondant de la série L, notamment pour l'acide glutamique, la méthionine, la leucine et la tyrosine.

Les dérivés bis-dinitrophénylés (cystine, tyrosine, lysine, histidine) exigent un nombre de millimoles de fluorodinitrobenzène double de celui de l'acide aminé. La quantité de carbonate de sodium est portée à 4 g dans le cas de la cystine et de la lysine et à 3 g dans le cas de la tyrosine, de l'histidine, de l'acide aspartique et de l'acide glutamique.

2. *Procédé de* RAO ET SOBER[69]

La réaction de condensation est réalisée en agitant l'acide aminé avec le fluorodinitrobenzène en présence d'un léger excès de bicarbonate de sodium pendant 2 à 5 h dans l'éthanol à 50% et à la température du laboratoire (il est recommandé d'effectuer les condensations ainsi que tous les stades de la préparation à l'obscurité). L'alcool est éliminé à la température du laboratoire et l'excès de fluorodinitrobenzène extrait par agitation trois fois avec de l'éther. La solution aqueuse est acidifiée jusqu'à réaction nettement acide par de l'acide chlorhydrique 6 *N*. Le précipité ou l'huile qui se sépare est lavé plusieurs fois avec de petites quantités d'eau glacée.

(*i*) *Cas des DNP-aminoacides précipités sous forme d'huile.* Procédé valable pour les acides aminés suivants de la série L: valine, alanine, acide α-aminobutyrique, norvaline, isovaline, thréonine, *allo*thréonine, leucine, isoleucine, *allo*isoleucine, hydroxyproline, phénylalanine, acide aspartique, cystine, ainsi que l'acide DL-glutamique, la DL-proline, la DL-thréonine, la DL-méthionine, la DL-éthionine, l'acide DL-α-aminobutyrique, la DL-valine, l'acide DL-pipécolique, la D-thréonine.

La substance est dissoute dans un grand volume d'acétone et l'on sèche la solution sur sulfate de sodium anhydre. Après filtration, on concentre la solution à un petit volume. On ajoute un volume égal de benzène à la solution acétonique et on précipite le DNP-aminoacide par un excès d'éther de pétrole (E: 30–75°). Le dérivé est séché sous un courant d'air, dissous dans l'éther et précipité avec de l'éther de pétrole. L'étape éther–éther de pétrole est répétée plusieurs fois jusqu'à ce que le DNP-aminoacide cristallise à basse température.

(*ii*) *Cas des DNP-aminoacides précipités sous forme solide.* Glutamine, L-sérine, L-tyrosine, L-tryptophanne, L-arginine, L-histidine, acide L-α,γ-diaminobutyrique, L-ornithine, L-lysine, acide γ-aminobutyrique et L-asparagine.

Les précipités sont lavés à l'eau glacée et cristallisés à partir de solvants appropriés (voir Tableau I).

b. Cas particuliers de synthèse

1. *Acide DNP-L-glutamique*

(*i*) *Procédé de* RAO ET SOBER[69]. A cause des difficultés de cristallisation, les auteurs partent de la DNP-glutamine, facilement cristallisable, qui est hydrolysée une nuit avec 10 fois le volume d'acide chlorhydrique 6 *N* et chauffée au bain-marie jusqu'à ce que le produit passe en solution. La solution refroidie à la température du laboratoire est placée ensuite dans un congélateur jusqu'à ce qu'une huile visqueuse jaune se sépare, qui cristallise après un séjour de plusieurs semaines dans le froid. Le DNP-aminoacide, lavé à l'eau et desséché sur P_2O_5 dans le vide, se présente sous forme d'un solide jaune hygroscopique.

(*ii*) *Procédé de* LEVY ET CHUNG[45]. L'acide L-glutamique (2.9 g) et le carbonate de sodium anhydre (6.0 g) sont dissous dans 100 ml d'eau à 40°. Le fluorodinitrobenzène (3.7 g) est ajouté et le mélange agité vigoureusement par un agiteur magnétique, la température étant maintenue à 40° pendant 30 min. Après acidification par de l'acide chlorhydrique concentré, l'acide DNP-glutamique recueilli est recristallisé du mélange chloroforme–acétate d'éthyle (F: 134–136°).

2. *Dérivés de l'histidine*

(*i*) *α-Mono-DNP-histidine* (composé présentant une réaction de Pauly positive et ne donnant pas la réaction à la ninhydrine). Le dérivé monosubstitué en position α de l'histidine s'obtient en faisant réagir 0.5 molécule (ou moins) de fluorodinitrobenzène et 1.0 molécule d'histidine. Le mode opératoire adopté par RAMACHANDRAN ET MCCONNELL[68] est le suivant: 1.917 g (0.01 mole) de monochlorhydrate de L-histidine et 8.4 g de bicarbonate de sodium sont dissous dans 200 ml d'eau et on ajoute 0.453 g (0.0025 mole) de fluorodinitrobenzène dans 25 ml d'éthanol. Après un contact de 1 h à la température du laboratoire, le volume est réduit sous vide à 50 ml et le pH ajusté à 6.5 par de l'acide chlorhydrique pur ajouté avec précaution. Le précipité est éliminé par filtration et recristallisé à partir d'une solution dans le volume minimum d'éthanol aqueux. Les cristaux jaunes et brillants obtenus après 18 h de séjour à basse température sont recueillis et séchés sous vide à 60°. F (non corrigé): 278–280° (décomposition).

(*ii*) *Di-DNP-histidine* (voir plus haut). Le dérivé dinitrophénylé s'obtient en faisant réagir un excès de fluorodinitrobenzène (2.5 moles par molécule d'histidine d'après RAMACHANDRAN ET McCONNELL[68]). Cristallisation à partir de l'acétone aqueuse.

(*iii*) *Imidazole-DNP-histidine* (composé non coloré donnant une coloration brune à la ninhydrine et ne présentant pas la réaction de Pauly). La synthèse est faite à partir de l'α-acétylhistidine (synthèse de BERGMANN ET ZERVAS[8]). Après dinitrophénylation, on hydrolyse l'α-acétyl-mono-DNP-imidazole-histidine par l'acide chlorhydrique à 20% à l'ébullition (MARGOLIASH[54]). On peut également obtenir l'imidazole-DNP-histidine après hydrolyse du peptide DNP-histidylhistidine. Mais l'hydrolysat contient également de la di-DNP-histidine et aussi parfois de l'α-mono-DNP-histidine.

3. *O-DNP-tyrosine* (synthèse de SANGER[77])

La N-acétyl-L-tyrosine (0.55 g) (DU VIGNEAUD ET MEYER[26]) est traitée en milieu bicarbonaté pendant 4 h avec 2.0 g de *chloro*dinitrobenzène dissous dans l'éthanol. Après acidification, il se sépare une huile qui cristallise partiellement. La N-acétyl-O-DNP-tyrosine est hydrolysée 3 h à reflux avec de l'acide chlorhydrique à 20%. Après refroidissement et évaporation à un petit volume, on obtient un précipité qui est séparé par filtration, dissous dans l'acide nitrique dilué chaud et neutralisé avec de la pyridine, tandis qu'il est encore chaud. L'O-DNP-tyrosine cristallise en aiguilles blanches contenant 1 molécule d'eau de cristallisation (F: 202°).

4. α-*Mono-DNP-arginine* (PORTER ET SANGER[67])

La condensation se fait en milieu éthanol aqueux. 3 moles d'acide aminé, 1.1 g de bicarbonate de sodium sont dissous dans 14 ml d'eau. On ajoute 1.1 g (6 mmoles) de fluorodinitrobenzène dans 28 ml d'éthanol. Le mélange est agité 2 h à la température du laboratoire et concentré pour éliminer l'éthanol.

Après enlèvement de l'éthanol du milieu réactionnel, le résidu est traité avec de l'eau: la DNP-arginine est insoluble. Après filtration, elle est lavée à l'éthanol et à l'éther. Elle est recristallisée à partir d'une solution d'acide chlorhydrique dilué, qui est neutralisée par l'ammoniaque.

5. *Dérivés de l'ornithine*

(*i*) α-*Mono-DNP-ornithine*. Après dinitrophénylation de la δ-benzoyl-ornithine (SANGER[77]), on hydrolyse l'α-DNP-δ-benzoyl-ornithine.

Le monochlorhydrate de DL-ornithine (0.3 g) (dérivé préparé à partir du bichlorhydrate par la méthode de RIVARD[73]) est converti en son complexe cuivrique (voir plus loin, paragraphe 5 (ii)) et la solution refroidie est benzoylée de la manière habituelle par 0.32 ml de chlorure de benzoyle et en présence de 6 ml de NaOH *N*. Le dérivé benzoylé insoluble est séparé par filtration, mis en suspension dans 2 ml d'eau et traité par l'hydrogène sulfuré. La solution est portée à l'ébullition et filtrée chaude. Après concentration du filtrat à 5 ml environ, la δ-benzoyl-DL-ornithine cristallise (0.14 g). Elle est mise en suspension dans 2 ml d'eau contenant 0.3 g de bicarbonate de sodium et agitée pendant 2 h avec une solution de 0.2 ml de fluorodinitrobenzène dans 4 ml d'éthanol. Après élimination de l'éthanol sous pression réduite, l'excès de fluorodinitrobenzène est enlevé par l'éther sulfurique et la solution est acidifiée. L'α-DNP-δ-benzoyl-DL-ornithine précipite immédiatement sous la forme d'un solide amorphe (rendement = 0.2 g). L'enlèvement du groupe benzoyle se fait par chauffage pendant 4 jours à 105° dans un tube scellé sous vide avec un mélange de 2 ml de HCl 10 *N* et de 2 ml d'acide acétique pur cristallisable. Après refroidissement, la solution est évaporée à siccité et le résidu dissous dans l'eau. Le produit non hydrolysé (quelquefois en quantité importante) est extrait par l'acétate d'éthyle et, après neutralisation de la solution aqueuse avec de la pyridine, l'α-DNP-ornithine cristallise (F: 227°).

Plutôt que d'avoir recours à la technique précédente, nous préférons dinitrophényler la δ-carbobenzoxy-DL-ornithine (voir paragraphe 6 (i), la préparation de l'ε-carbobenzoxy-lysine) et enlever le groupement carbobenzoxy par hydrolyse par l'acide chlorhydrique 5.6 *N* à 100° pendant 24 h.

(*ii*) *δ-DNP-L-ornithine.* Elle se prépare par dinitrophénylation du complexe cuivrique du monochlorhydrate de la L-ornithine (SANGER[77]).

0.1 g du monochlorhydrate de L-ornithine est dissous dans 5 ml d'eau chaude et traité par un excès de carbonate de cuivre. Après filtration, la solution est évaporée jusqu'à un volume d'environ 2 ml et on ajoute 0.3 g de bicarbonate de sodium et une solution de 0.2 ml de fluorodinitrobenzène dans 4 ml d'éthanol. Le mélange est agité pendant 2 h à la température du laboratoire. Le complexe cuivrique de la δ-DNP-ornithine précipite sous la forme d'une poudre verdâtre. Après filtration et dissolution dans l'acide chlorhydrique dilué, la solution est traitée par de l'hydrogène sulfuré et filtrée sur charbon. Après concentration, le chlorhydrate de δ-DNP-L-ornithine cristallise. Il est recristallisé à partir d'une solution dans l'acide chlorhydrique *N* (F: 228°, décomposition).

(*iii*) *Di-DNP-ornithine.* Elle s'obtient facilement par les méthodes classiques (excès de fluorodinitrobenzène).

6. *Dérivés de la lysine*

(*i*) *α-Mono-DNP-L-lysine.* Elle peut être obtenue par dinitrophénylation de l'ε-benzoyl-lysine, de l'ε-acétyl-lysine ou de l'ε-carbobenzoxy-lysine, suivie d'une hydrolyse spécifique du groupe protecteur du radical ε-aminé.

ε-Benzoyl-lysine (SANGER[76]). La méthode est peu commode.

ε-Acétyl-lysine. Ce dérivé est préparé par la méthode de NEUBERGER ET SANGER[61]. Après dinitrophénylation de l'ε-acétyl-lysine, l'α-DNP-ε-acétyl-lysine est extraite par l'éther de la solution acide. Le déblocage du groupe acétyl se fait par l'acide chlorhydrique 6 *N* pendant 2 h sous pression ("15 lb. steam pressure"). L'acide est chassé sous pression réduite et l'α-DNP-lysine est précipitée par la pyridine (FOLK[27]) (F: 270°, décomposition).

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ε-Carbobenzoxy-L-lysine. Ce dérivé est préparé soit par la méthode de BERGMANN et coll.[9], soit par la méthode de NEUBERGER ET SANGER[61].

Le monochlorhydrate de lysine (1.8 g) (préparé à partir du dichlorhydrate par la méthode décrite par RICE[72]) est traité en solution aqueuse à chaud par un excès de carbonate de cuivre. L'excès de carbonate de cuivre est éliminé par filtration. On ajoute 5 ml de soude 2 *N* et la solution bleu foncé est refroidie dans la glace. Le chlorure de carbobenzoxy (2 ml) et la soude 2 *N* (10 ml) sont ajoutés en 10 portions pendant 30 min sous agitation, en refroidissant et en prenant soin que la solution ne devienne trop alcaline. Le complexe cuivrique de l'ε-carbobenzoxy-lysine précipite. Après filtration, il est lavé à l'éthanol et à l'eau et mis en suspension dans 20 ml d'eau. On fait passer un courant d'hydrogène sulfuré. La solution portée à l'ébullition est filtrée chaude et l'ε-carbobenzoxy-lysine cristallise en fines aiguilles. On la recristallise de sa solution aqueuse.

La dinitrophénylation de l'ε-carbobenzoxy-lysine s'effectue par les méthodes habituelles et l'hydrolyse de l'α-DNP-ε-carbobenzoxy-lysine est réalisée en milieu chlorhydrique 5.6 *N* pendant 16 h, à 100°.

(*ii*) *ε-Mono-DNP-lysine.* Sa préparation se fait par dinitrophénylation du complexe cuivrique du monochlorhydrate de L-lysine (PORTER ET SANGER[67]).

La L-lysine (0.5 g) est dissoute dans 10 ml d'eau et le carbonate de cuivre est ajouté lentement à la solution bouillante. L'excès de carbonate de cuivre est éliminé par filtration. On ajoute un excès de bicarbonate de sodium et une solution de 1.5 g de fluorodinitrobenzène dans 20 ml d'éthanol. Le mélange est agité 2 h à la température du laboratoire. Le précipité jaune verdâtre est recueilli par filtration, lavé à l'eau, à l'éthanol et à l'éther. Il est mis en suspension dans 5 ml d'eau et une quantité suffisante d'acide chlorhydrique *N* est ajoutée pour obtenir une solution claire. La solution est refroidie dans la glace et on fait passer un courant d'hydrogène sulfuré pendant 2 min. On ajoute une trace de charbon et le mélange est immédiatement filtré. Le filtrat est évaporé rapidement à siccité sous pression réduite. Le produit est cristallisé à partir de sa solution dans l'acide chlorhydrique à 20%.

(*iii*) *Di-DNP-lysine.* Elle est obtenue facilement par les méthodes classiques (voir procédé de LEVY ET CHUNG, paragraphe B, a, 1, p. 226). Elle est recristallisée du méthanol aqueux.

7. *Dérivés de la cystine*

(*i*) *Mono-DNP-cystine* (BETTELHEIM[10]). 1.2 g de L-cystine et 2 g de carbonate de sodium sont dissous dans 50 ml d'eau; on ajoute 0.9 g de fluorodinitrobenzène dissous dans 5 ml d'éthanol. Après 20 min d'agitation, le pH est ajusté à 7 par de l'acide chlorhydrique et la cystine non dinitrophénylée précipite. La solution est de nouveau acidifiée et concentrée. Le chlorure de sodium est précipité par l'acétone. Après dessication, le résidu est lavé à l'éther et cristallisé de sa solution aqueuse (F: 187°, non corrigé).

(*ii*) *Di-DNP-cystine.* Elle est préparée par les méthodes classiques. Sa cristallisation peut se faire à partir de l'éther monoéthylique de l'éthylène-glycol aqueux. On la recristallise de sa solution dans l'acide acétique dilué (PORTER ET SANGER[67]).

(*iii*) *S-DNP-cystéine.* Après dinitrophénylation du glutathion réduit, l'hydrolysat total contient la S-DNP-cystéine (hydrosoluble) et l'acide DNP-glutamique (éthérosoluble) (HAUSMANN, WEISIGER ET CRAIG[32]).

(*iv*) *Acide DNP-cystéique* (sel de potassium). Pour obtenir ce dérivé dinitrophénylé hydrosoluble, il est commode d'oxyder par l'acide performique la di-DNP-cystine (BETTELHEIM[10]).

On oxyde 0.2 g de di-DNP-L-cystine par 10 ml d'acide performique (9 ml d'acide formique pur + 1 ml d'eau oxygénée à 110 volumes) pendant 30 min. La solution est évaporée à siccité, et le résidu est dissous dans un peu d'eau. La solution est ajustée à pH 6 avec de la potasse et cristallise par addition d'éthanol et d'éther.

C. DINITROPHÉNYLATION D'UN HYDROLYSAT DE PROTÉINE (Tableau II)

a. Hydrolyse de la protéine

3 à 5 mg de protéine sont hydrolysés en tube scellé sous vide par 1 à 2 ml d'acide chlorhydrique 5.7 *N*[*] à 105° pendant 24 h[**]. L'acide chlorhydrique de l'hydrolysat est très soigneusement éliminé.

b. Condensation avec le fluorodinitrobenzène; extraction des dérivés dinitrophénylés

Différentes modalités de condensation des acides aminés avec le fluorodinitrobenzène ont été décrites. On peut effectuer la réaction soit en milieu aqueux, soit en milieu hydro-alcoolique.

1. *Réaction de condensation en milieu aqueux* (LEVY *et al.*[46])

(*i*) *Conduite de la réaction.* L'hydrolysat (3 à 5 mg dans 3 ml) est placé dans une cellule de synthèse (voir Fig. 1). On ajoute 0.1 ml de chlorure de potassium 3.1 *N* et le contenu est ajusté à pH 9.0 (environ 40 μmoles de NaOH 0.2 *N*). La solution est saturée en fluorodinitrobenzène à 40° par agitation vigoureuse avec un léger excès (environ 0.1 ml) de réactif. Le pH est maintenu à 9 pendant 80 min par des additions intermittentes de soude 0.2 *N*. Cette opération peut être effectuée commodément au moyen d'un autotitrateur de JACOBSEN ET LEONIS[37].

La cinétique de la réaction peut être suivie en mesurant la consommation de soude (Fig. 2). Étant donné que le fluorodinitrobenzène est en excès, le milieu réactionnel reste saturé en réactif et la formation de dinitrophénol[***] est constante dans

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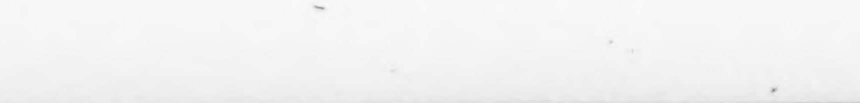
[*] L'acide chlorhydrique est préparé en distillant 3 à 4 fois dans un appareil en verre un mélange azéotropique d'acide chlorhydrique et d'eau.

[**] Habituellement la durée de l'hydrolyse est de 24 h; il peut être intéressant d'effectuer le dosage des acides aminés sur plusieurs séries d'hydrolysats obtenus à des temps différents (24 et 48 h, par exemple).

[***] Outre la réaction de condensation avec les acides aminés [FDNB + H_2N-CH(R)-COOH → DNP-HN-CH(R)-COOH + HF], il se produit également une hydrolyse alcaline du réactif



(dinitrophénol)

le temps (0.044 μmole/ml/min) (LEVY[43]). L'extrapolation au temps zéro de la pente finale de la courbe linéaire qui résulte de la formation du dinitrophénol indique le nombre de μmoles de soude consommées au cours de la réaction de condensation avec les acides aminés (Fig. 2).

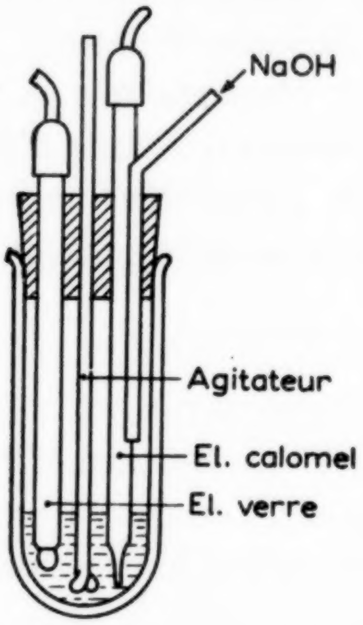


Fig. 1. Cellule de synthèse pour le dinitrophénylation des acides aminés. El. verre = électrode de verre; El. calomel = électrode au calomel.

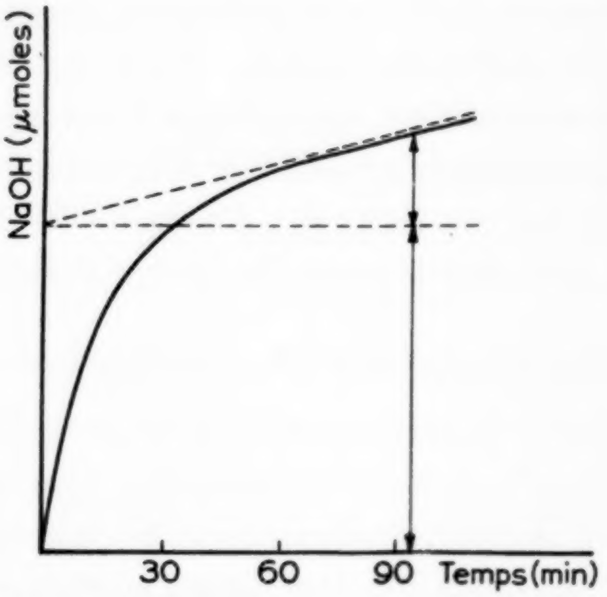


Fig. 2. Étude graphique de la consommation de soude au cours de la dinitrophénylation. L'extrapolation au temps zéro de la pente finale de la courbe, qui résulte de la formation du dinitrophénol, indique le nombre de μmoles de soude consommées au cours de la réaction de condensation avec les acides aminés.

FRAENKEL-CONRAT ET SINGER[29] ont décrit également une réaction de condensation dans du tampon carbonate à 5% de pH 9.3 pendant 3 h à 40°. La quantité de fluorodinitrobenzène est de 15 μl pour 2 mg d'acides aminés.

(*ii*) *Extraction des dérivés dinitrophénylés.* Lorsque la réaction de condensation est terminée, le contenu de la cellule est versé quantitativement dans une ampoule à décanter et extrait avec de l'éther privé de peroxydes* (2 à 4 fois 5 ml) pour éliminer l'excès de fluorodinitrobenzène. Ensuite, le mélange est acidifié (0.5 ml de HCl 5 *N*) et les DNP-aminoacides éthérosolubles sont extraits par de l'éther (5 fois 5 ml). LEVY[43] prélève des parties aliquotes de la solution éthérée (3 parties de 2 ml) qui sont évaporées à sec. Les résidus secs sont dissous dans l'acétone et leur solution est déposée sur des feuilles de papier Whatman No. 1. Nous préférons le mode opératoire suivant.

La phase éthérée est concentrée soigneusement dans des capsules de verre jusqu'à un volume réduit; la couche éthérée, qui surnage au-dessus de quelques gouttelettes d'eau, est versée quantitativement dans des récipients spéciaux (Fig. 3) en vue de la sublimation du dinitrophénol (technique personnelle dérivée de celle de MILLS, voir plus loin).

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* Pour éviter la transformation de la DNP-méthionine en DNP-méthionine-sulfone, il est indispensable d'employer de l'éther privé de peroxydes. On peut le distiller sur du chlorure stanneux, puis on le lave avec une solution de carbonate de sodium à 20% et avec de l'eau; on le conserve sur du sulfate ferreux pulvérisé, à l'abri de la lumière. On peut aussi priver l'éther de peroxydes par passage sur une colonne d'alumine activée (DASLER ET BAUER[21]).

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La phase aqueuse restant après l'extraction des DNP-aminoacides éthérosolubles contient encore l'α-mono-DNP-arginine et l'α-DNP-histidine (et éventuellement l'acide DNP-cystéique)*. Elle peut être traitée de différentes façons.

D'après LEVY[43], la phase aqueuse est diluée à 10 ml et des parties aliquotes (3 fois 1 ml) sont évaporées à sec. Après dissolution dans un volume connu d'acétone, la solution de DNP-aminoacides est déposée sur des feuilles de papier Whatman No. 1.

Nous préférons, comme KOCH ET WEIDEL[39], extraire plusieurs fois la phase aqueuse restante par un mélange à volume égal de *sec.*-butanol et d'acétate d'éthyle. Après dessication, les DNP-aminoacides hydrosolubles sont dissous dans un volume connu (3 ml, par exemple) d'acétone**. Des parties aliquotes de cette solution (50 à 150 μl) sont déposées sur du papier Whatman No. 1.

2. *Réaction de condensation en milieu hydro-alcoolique*

Pour obtenir la transformation totale de l'histidine en di-DNP-histidine, en évitant la formation d'α-mono-DNP-histidine (voir plus haut), FRAENKEL-CONRAT ET SINGER[29] réalisent la condensation avec le fluorodinitrobenzène en milieu hydro-alcoolique suivant les modalités techniques suivantes.

La condensation s'effectue dans un milieu contenant 0.7% de bicarbonate de sodium, 1.7% de fluorodinitrobenzène et 67% d'éthanol, pendant 80 min à 20–25°. L'excès de fluorodinitrobenzène est extrait à l'éther après évaporation de la plus grande partie de l'alcool. Le milieu réactionnel est ensuite acidifié (aux environs de pH 1–2) et extrait d'abord à l'éther privé de peroxydes, puis à l'acétate d'éthyle qui permet l'extraction quantitative de la di-DNP-histidine. La phase aqueuse restante ne contient que l'α-mono-DNP-arginine et éventuellement l'acide DNP-cystéique.

Nous préférons utiliser le mode opératoire suivant.

L'hydrolysat (10 mg de protéine) est dissous dans 5 ml d'eau bidistillée amenée et maintenue à 40°. On ajuste à pH 9 par de la soude $N/15$ et on ajoute 0.2 ml de fluorodinitrobenzène. On agite pendant 15 min à 40° en maintenant le pH à 9. On ajoute alors 10 ml d'éthanol absolu et on continue l'agitation pendant 90 min à 40° en maintenant toujours le pH à 9. Après la condensation, l'alcool est chassé par ventilation d'air froid. L'excès de fluorodinitrobenzène est extrait plusieurs fois (5 à 10 fois) par de l'éther privé de peroxydes. Le milieu est acidifié (1 ml d'acide chlorhydrique concentré pur) et extrait de nouveau par l'éther privé de peroxydes (5 extractions) puis par l'acétate d'éthyle (3 extractions). La phase aqueuse résiduelle est extraite par le mélange à parties égales d'acétate d'éthyle et de *sec.*-butanol (3 extractions).

Les extractions par l'éther (en milieu acide) et par l'acétate d'éthyle sont rassemblées et évaporées à sec, et le dinitrophénol contenu dans cette phase est éliminé par sublimation (voir plus loin la description de la technique employée). Les DNP-aminoacides sont dissous dans 2 ml d'acétone.

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* L'acide DNP-cystéique peut se trouver dans les hydrolysats totaux de protéine oxydée par l'acide performique ou dans les hydrolysats totaux oxydés par l'acide performique.

** Pour faciliter la dissolution des DNP-aminoacides hydrosolubles on peut utiliser de l'acétone acide (acétone 0.9 ml + acide chlorhydrique N 0.1 ml).

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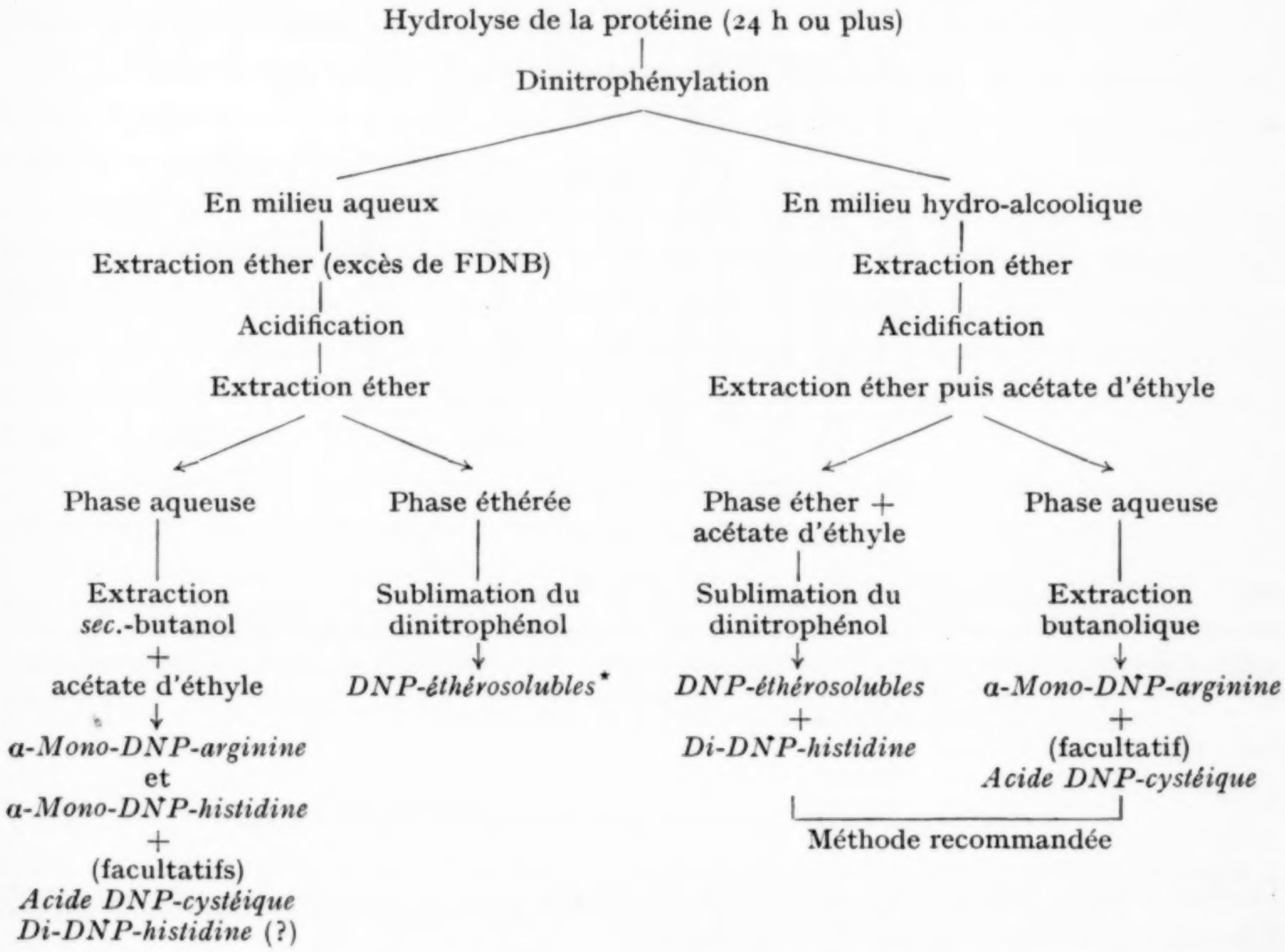
TABLEAU II

DOSAGE DES ACIDES AMINÉS SOUS LA FORME DE LEURS DÉRIVÉS DINITROPHÉNYLÉS

(Méthode de LEVY[43])


Hydrolyse de la protéine (24 h ou plus)
Dinitrophénylation
En milieu aqueux
Extraction éther (excès de FDNB)
Acidification
Extraction éther
Phase aqueuse
Extraction
sec.-butanol
+
acétate d'éthyle
a-Mono-DNP-arginine
et
a-Mono-DNP-histidine
+
(facultatifs)
Acide DNP-cystéique
Di-DNP-histidine (?)
Phase éthérée
Sublimation du dinitrophénol
*DNP-éthérosolubles**
En milieu hydro-alcoolique
Extraction éther
Acidification
Extraction éther puis acétate d'éthyle
Phase éther + acétate d'éthyle
Sublimation du dinitrophénol
DNP-éthérosolubles
+
Di-DNP-histidine
Phase aqueuse
Extraction butanolique
a-Mono-DNP-arginine
+
(facultatif)
Acide DNP-cystéique
Méthode recommandée

* *Remarque*: On fera attention à la di-DNP-histidine, qui peut accompagner en très faible quantité les DNP-aminoacides éthérosolubles.

Les extraits butanoliques sont également évaporés à sec, puis dissous dans l'acétone (1 à 2 ml). Des parties aliquotes (correspondant à 0.1–0.3 mg de protéine) des solutions acétoniques des résidus des DNP-éthéro- et hydrosolubles sont déposées sur des feuilles de papier Whatman No. 1.

c. Sublimation du dinitrophénol

L'élimination du dinitrophénol se fait classiquement par sublimation (MILLS[58]). Le dispositif de sublimation que nous utilisons est représenté sur la Fig. 3. Cet appareil est conçu pour que la distance entre le film chauffé de dérivés dinitrophénylés et la surface de verre refroidie (neige carbonique + acétone dans le réfrigérant) soit très courte.

Les extraits éthérés sont placés dans les fioles spéciales et évaporés à siccité. Si, après évaporation de l'éther, il reste une gouttelette d'eau dans la fiole, on place un chapeau de verre (sans tube réfrigérant) sur le rodage de la fiole. L'eau est facilement éliminée sous pression réduite en quelques minutes. La sublimation du dinitrophénol s'effectue à 70–80° (les fioles ne sont plongées dans le bain-marie qu'après l'établissement du vide) pendant 30 min*.

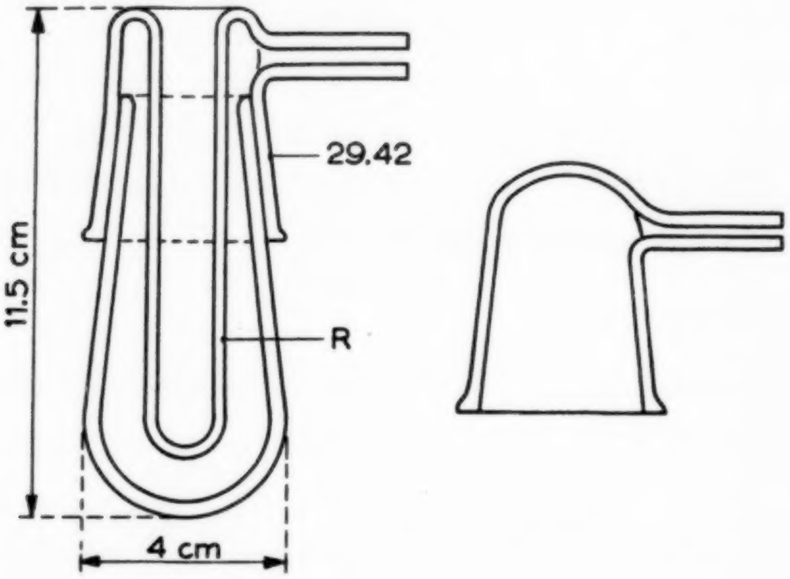


Fig. 3. Appareil employé pour la sublimation du dinitrophénol. Le joint conique de l'appareil est un rodage normalisé 29–42. Le réfrigérant (R) est rempli par le mélange acétone + neige carbonique. Le chapeau de verre sans refrigérant (partie droite de la figure) permet l'élimination des gouttelettes d'eau après l'évaporation de la solution éthérée et avant la sublimation elle-même.

D. DINITROPHÉNYLATION D'UNE PROTÉINE (Tableau III)

La dinitrophénylation d'une protéine à été décrite pour la première fois par SANGER[76]. Plusieurs procédés peuvent être employés.

a. Technique de condensation avec le fluorodinitrobenzène

La condensation peut s'effectuer sur les protéines natives, dénaturées ou oxydées.

Avec les protéines natives, un petit nombre de groupements ε-aminés des résidus intrapeptidiques de lysine et de noyaux imidazole de résidus d'histidine peuvent ne pas être substitués par le fluorodinitrobenzène (PORTER[65]). Cette absence de réactivité ne gêne nullement la détermination des résidus d'aminoacides en position N-terminale.

Dans le cas de la cystine ou d'un demi-résidu de cystine en position N-terminale (cas du chymotrypsinogène, BETTELHEIM[10]), il est indispensable de faire l'identification sous la forme d'acide DNP-cystéique. On peut pour cela dinitrophényler la protéine oxydée ou oxyder la DNP-protéine. L'oxydation de la protéine doit être réalisée d'une façon très douce, notamment par les procédés de THOMPSON[87] et de HIRS[34]**.

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* Au cours de la sublimation, la DNP-méthionine peut être légèrement entraînée avec le dinitrophénol.

** Pour éviter la destruction des DNP-aminoacides, il est indispensable d'éliminer toute trace d'eau oxygénée. On peut obtenir ce résultat par précipitation de la DNP-protéine oxydée par l'éther privé de peroxydes, suivie par une dissolution dans l'acide formique et une reprécipitation à l'éther. Cette opération est répétée 3 fois.

1. *Condensation en milieu éthanol–bicarbonate* (SANGER[76])

La protéine (0.5 g par exemple) et le bicarbonate de sodium (0.5 g) sont dissous dans 5 ml d'eau. On ajoute à la solution 10 ml d'une solution éthanolique à 5% (v/v) de fluorodinitrobenzène et le mélange est agité mécaniquement pendant 2 à 3 h à l'obscurité et à la température du laboratoire.

La dinitrophénylation d'une protéine insoluble nécessite la prolongation du temps d'agitation (48 h à 40°, 72 h à 20°) et des additions répétées de bicarbonate de sodium et de fluorodinitrobenzène.

Après sa dinitrophénylation totale, la DNP-protéine est souvent insoluble, même en milieu alcalin; d'autre part, après acidification du milieu, la plupart des DNP-protéines précipitent. Après centrifugation, le précipité est lavé plusieurs fois à l'eau (pour enlever les sels minéraux), à l'alcool jusqu'à obtention d'un liquide surnageant incolore (pour éliminer l'excès de fluorodinitrobenzène et le dinitrophénol formé) et finalement à l'éther sulfurique.

La conduite de ces étapes de lavage peut être modifiée suivant les solubilités particulières des DNP-protéines (solubilité dans l'eau de la DNP-salmine et du DNP-ovocomucoïde, qui sont précipitables par un excès d'éthanol, solubilité dans l'eau et dans l'alcool de la DNP-glycoprotéine acide α_1 du sérum ou DNP-orosomucoïde). L'élimination des réactifs ou des artefacts de condensation peut toujours être obtenue par dialyse et la solution de DNP-protéine peut être lyophilisée.

2. *Condensation en milieu aqueux*

LEVY ET LI[47] ont décrit une réaction de condensation en milieu aqueux maintenu à pH 8 à l'aide d'un auto-titrateur (modèle JACOBSEN-LEONIS, par exemple).

La protéine (0.2 μmole au minimum) est dissoute dans 3 ml de chlorure de potassium 0.1 M à 40°. Le pH est ajusté et maintenu à 8 par des additions de potasse 0.05 N. Après l'addition du fluorodinitrobenzène (0.1 ml environ), la solution est agitée vigoureusement. La réaction de condensation est terminée après 90 à 120 min d'agitation: la courbe de consommation de la potasse en fonction du temps s'infléchit lorsque la réaction se termine; la pente constante de la courbe correspond alors à l'hydrolyse du fluorodinitrobenzène.

La DNP-protéine peut rester soluble dans le milieu réactionnel, qui est alors extrait 3 fois à l'éther pour enlever l'excès de fluorodinitrobenzène. Après acidification, la DNP-protéine précipite. Elle est recueillie par centrifugation, lavée à l'eau, à l'acétone et à l'éther, et séchée sur P_2O_5.

3. *Condensation en milieu bicarbonate–chlorhydrate de guanidine*

D'après PHILLIPS[64], on obtient un rendement plus satisfaisant en groupes terminaux en réalisant la dinitrophénylation en milieu bicarbonate de potassium et chlorhydrate de guanidine. Le mode opératoire préconisé est le suivant.

La protéine est dissoute dans une solution de chlorhydrate de guanidine 6 M (concentration 20 mg de protéine/ml). On ajoute du bicarbonate de potassium solide à la concentration de 10–15 mg/ml et du fluorodinitrobenzène à la concentration de 0.05–0.1 ml/ml. Le mélange est agité à 20° pendant un temps qui peut varier de 6 à 24 h. Il est ensuite acidifié, dilué avec 3 volumes d'eau et extrait une fois à l'éther, les deux phases étant facilement séparées par centrifugation.

Le précipité de DNP-protéine est lavé à l'eau plusieurs fois par centrifugation. Les dernières traces de réactif et de dinitrophénol sont éliminées par trois lavages à l'acétone et un lavage à l'éther.

b. Détermination de la quantité de protéine dans la DNP-protéine

Pour déterminer le nombre de molécules d'acides aminés en position N-terminale par molécule de protéine, il est indispensable de connaître exactement la quantité de protéine contenue dans la DNP-protéine. En première approximation, 80% du poids de DNP-protéine correspondent à la protéine initiale.

Avec les procédés de synthèse décrits plus haut (notamment le procédé de condensation en milieu éthanol–bicarbonate), il est possible d'opérer soigneusement et quantitativement et de tenir compte exclusivement du poids de protéine mis en œuvre au moment de la réaction de condensation.

Il est beaucoup plus commode de doser, dans la protéine et dans la DNP-protéine obtenue, un groupement ou un résidu qui n'a pas été modifié par la réaction de condensation: le groupement amide ne réagit pas avec le fluorodinitrobenzène et peut être dosé simplement et d'une façon précise.

Selon SANGER[76], la DNP-protéine est hydrolysée dans l'acide chlorhydrique 2 *N* bouillant pendant 4 h. Après neutralisation, l'ammoniaque libérée est distillée dans un appareil de microkjeldahl à 95°, en employant un tampon borate de pH 9.5.

Selon THOMPSON[85], la DNP-protéine est hydrolysée 9 h à 105° en tube scellé avec de l'acide chlorhydrique 2 *N*. Après neutralisation avec de la soude 2 *N* à pH 5 (vert de bromocrésol), l'ammoniaque est distillée après addition de tampon phosphate 0.15 *M* de pH 10.5.

Signalons également que le poids moléculaire de la DNP-protéine peut être calculé lorsque le nombre total de résidus d'acides aminés de la protéine est entièrement connu, en supposant que les résidus intrapeptidiques de lysine, de tyrosine, d'histidine, de cystéine et tous les résidus en position N-terminale ont été substitués. Il suffit d'ajouter au poids moléculaire de la protéine la valeur obtenue en multipliant par 166 le nombre total de radicaux dinitrophényle introduits.

c. Hydrolyse de la DNP-protéine

1. *Hydrolyse totale de la DNP-protéine*

Les conditions d'hydrolyse totale de la protéine, en vue de la détermination du résidu d'acide aminé en position N-terminale, varient suivant les substrats et suivant la nature de l'acide aminé terminal. Il est nécessaire que l'hydrolyse soit assez poussée pour que l'hydrolysat ne contienne pas (ou peu) de DNP-peptides et qu'elle ne soit pas trop longue pour éviter au maximum la destruction du DNP-aminoacide terminal (pour le taux de destruction des DNP-aminoacides au cours de l'hydrolyse, voir: Chromatographie quantitative, paragraphe J, p. 269).

Pour la détermination qualitative du résidu N-terminal, l'hydrolyse s'effectue habituellement en tube scellé sous vide (ou sous azote) pendant 16 h à 105° en présence d'acide chlorhydrique 5.7 *N* redistillé (voir: Hydrolyse de la protéine, paragraphe C, a, p. 232).

Ce type d'hydrolyse peut toutefois provoquer une destruction importante de la DNP-proline et du DNP-glycocolle.

L'hydrolyse de la DNP-proline pendant 16 h par de l'acide chlorhydrique 12 *N* entraîne une destruction de plus de 50%*. Au cours de l'hydrolyse, la DNP-proline peut former, par rupture du noyau, deux dérivés particuliers, l'acide α-chloro-δ-DNP-aminovalérique et l'acide δ-chloro-α-DNP-aminovalérique (voir paragraphe G, a, 5, p. 253).

Après 8 h d'hydrolyse par l'acide chlorhydrique 5.7 *N*, 60% du DNP-glycocolle sont détruits (PORTER[66]).

Pour éviter au maximum ces destructions, il est recommandé d'effectuer, en plus de l'hydrolyse à 105° pendant 16 h, deux autres hydrolyses:

pour le DNP-glycocolle, hydrolyse par l'acide chlorhydrique 5.7 *N* 4 h; dans ces conditions, il est indispensable de préciser si l'hydrolysat contient des DNP-peptides;

pour la DNP-proline, hydrolyse pendant 4 h par de l'acide chlorhydrique 11.2 *N* (PORTER ET SANGER[67], PHILLIPS[64]). SHEPHERD *et al.*[83] conseillent également une hydrolyse de 24 h à 105° par un mélange à parties égales d'acide acétique et d'acide chlorhydrique.

La destruction des DNP-aminoacides au cours de l'hydrolyse acide est très nettement augmentée en présence de tryptophanne ou de protéines riches en tryptophanne comme le lysozyme (THOMPSON[84]). La xanthylation du tryptophanne ou de la protéine réduit très nettement le taux de destruction (DICKMAN ET ASPLUND[25]), le dixanthyltryptophanne étant stable à l'hydrolyse acide. Il y a donc parfois intérêt à réaliser la xanthylation de la DNP-protéine. La DNP-protéine est dissoute dans de l'acide acétique à 90% en présence de xanthydrol. Après une heure de contact à la température du laboratoire, la xanthyl-DNP-protéine est précipitée par l'éther et lavée à l'éther par centrifugation.

2. *Hydrolyse partielle acide de la DNP-protéine*

Il y a intérêt parfois à hydrolyser partiellement la DNP-protéine de façon à isoler et à déterminer la structure des DNP-peptides de la séquence N-terminale. Cette hydrolyse partielle peut s'obtenir par l'action de l'acide chlorhydrique 12 *N* à 37° ou de l'acide chlorhydrique 5.6 *N*, 3 *N* et 0.1 *N* à 100° pendant des temps variables.

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* Une hydrolyse par l'acide acétique à 96% pendant 16 h à 100° détruit également 50% de la DNP-proline. Dans ces conditions, la DNP-protéine n'est pas totalement hydrolysée (SCANES ET TOZER[81]).

3. *Hydrolyse enzymatique de la DNP-protéine*

Les enzymes protéolytiques peuvent hydrolyser partiellement certaines DNP-protéines. Habituellement la vitesse de réaction est nettement réduite et le degré d'hydrolyse sensiblement abaissé. L'hydrolyse enzymatique permet également d'aborder le problème de la structure des DNP-peptides de la séquence N-terminale. Des exemples intéressants ont été décrits: par exemple, hydrolyse pepsique de la DNP-ribonucléase (ANFINSEN *et al.*[2]), hydrolyse trypsique spécifique de la DNP-ribonucléase oxydée (REDFIELD ET ANFINSEN[70]), hydrolyse par la carboxypeptidase d'une DNP-protéine en vue de la détermination de la séquence C-terminale (WALDSCHMIDT-LEITZ ET GAUSS[90]).

d. Extraction des dinitrophénylaminoacides

1. *Extraction à partir d'un hydrolysat total de protéines*

Après l'hydrolyse, les hydrolysats sont dilués avec de l'eau pour obtenir une concentration sensiblement normale en acide chlorhydrique. L'hydrolysat dilué est extrait 4 fois par 5 ml d'éther privé de peroxydes (voir note p. 233). Les extraits éthérés sont lavés avec de l'acide chlorhydrique 0.1 *N* (PHILLIPS[64]) ou bien 3 fois à l'eau (THOMPSON[85]) pour éliminer toute trace des dérivés acidosolubles (comme l'ε-mono-DNP-lysine) qui peuvent avoir été entraînés au cours de l'extraction. Les extraits éthérés sont concentrés et le dinitrophénol qui les souille est éliminé par sublimation suivant les modalités techniques décrites plus haut (paragraphe C, c, p. 235). Les DNP-aminoacides éthérosolubles sont dissous dans un volume déterminé d'acétone. Une partie aliquote de cette solution est déposée sur des feuilles de papier Whatman No. 1. Les DNP-aminoacides éthérosolubles peuvent contenir des traces de di-DNP-histidine lorsque celle-ci existe en position N-terminale. Une extraction continue à l'éther dans un appareillage spécial (MILLS[58]) permet d'obtenir également ce dérivé dans la fraction éthérosoluble. Une extraction par l'acétate d'éthyle, après l'extraction éthérée, conduit aussi à l'isolement de ce composé, mais il faut se rappeler que ce dernier solvant peut également extraire des DNP-peptides éventuellement présents (voir paragraphe D, d, 2, p. 242).

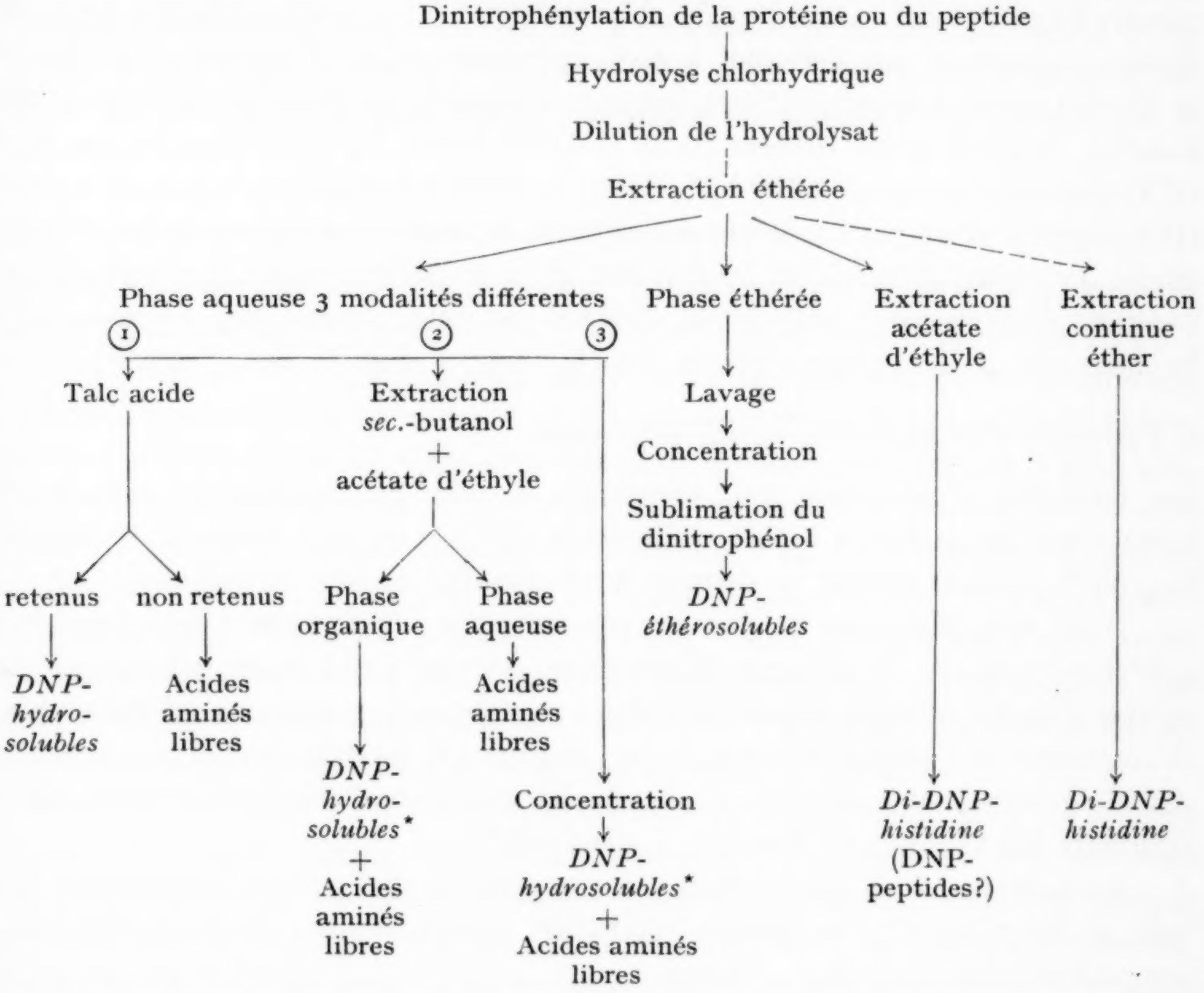
La phase aqueuse résiduelle de l'hydrolysat contient tous les aminoacides libres, toujours des DNP-aminoacides hydrosolubles comme l'ε-mono-DNP-lysine, l'O-DNP-tyrosine (incolore), l'imidazole-DNP-histidine et éventuellement la S-DNP-cystéine, qui proviennent des résidus correspondants d'aminoacides en position intrapeptidique. On peut également trouver l'α-mono-DNP-arginine, l'acide DNP-cystéique (hydrolysat oxydé de protéine ou hydrolysat de protéine oxydée), la di-DNP-histidine (si l'hydrolysat n'a pas été extrait d'une façon continue à l'éther, ou par la combinaison successive éther et acétate d'éthyle), l'α-mono-DNP-histidine, lorsque ces résidus d'acides aminés existent en position N-terminale dans la protéine.

Les dérivés dinitrophénylés hydrosolubles peuvent être extraits (mais ce n'est pas nécessaire) par le *sec.*-butanol. Les phases organiques ainsi obtenues et la phase aqueuse restante sont évaporées à sec, reprises dans un volume connu d'acétone

TABLEAU III

DÉTERMINATION DE L'ACIDE AMINÉ PORTEUR D'UN GROUPE α-AMINÉ TERMINAL LIBRE

(Méthode de SANGER[76])


Dinitrophénylation de la protéine ou du peptide
Hydrolyse chlorhydrique
Dilution de l'hydrolysat
Extraction éthérée
Phase aqueuse 3 modalités différentes
①
②
③
Phase éthérée
Extraction acétate d'éthyle
Extraction continue éther
Talc acide
Extraction sec.-butanol + acétate d'éthyle
Lavage
Concentration
Sublimation du dinitrophénol
DNP-éthérosolubles
retenus
non retenus
Phase organique
Phase aqueuse
DNP-hydrosolubles
Acides aminés libres
Acides aminés libres
DNP-hydrosolubles* + Acides aminés libres
Concentration
DNP-hydrosolubles* + Acides aminés libres
Di-DNP-histidine (DNP-peptides?)
Di-DNP-histidine

* Les DNP-hydrosolubles contiennent:

(1) *Dérivés provenant d'acides aminés non-terminaux*: ε-mono-DNP-lysine, imidazole-DNP-histidine (incolore), O-DNP-tyrosine (incolore), S-DNP-cystéine (incolore); éventuellement (peptides bactériens) δ-mono-DNP-ornithine et acide γ-mono-DNP-diaminobutyrique.

(2) *Dérivés provenant d'acides aminés terminaux*: α-mono-DNP-arginine, di-DNP-histidine, α-mono-DNP-histidine, acide DNP-cystéique; cas particuliers: α-mono-DNP-lysine, α-mono-DNP-ornithine, acide α-mono-DNP-diaminobutyrique.

(légèrement acide si c'est nécessaire) et étudiées en chromatographie de partage sur papier. La séparation des dérivés dinitrophénylés de la phase aqueuse et des acides aminés libres (tous les acides aminés constitutifs plus quelques molécules de lysine et d'histidine "non réactives") peut être obtenue sur une colonne de talc (SANGER[78]). Le résidu aqueux est dissous dans 2 ml d'acide chlorhydrique *N* et passé sur une colonne (2.5 cm de diamètre) qui contient un mélange de 20 g d'Hyflo-Super-Cel et 50 g de talc* imbibé d'acide chlorhydrique *N*. Les acides aminés libres ne sont pas adsorbés tandis que tous les dérivés dinitrophénylés (sauf l'acide cystéique, REDFIELD ET ANFINSEN[70]) le sont. Après un lavage soigneux de la colonne avec de l'acide chlorhydrique (100 ml), les dérivés dinitrophénylés sont élués par 400 ml d'alcool chlorhydrique (alcool: 4 vol., acide chlorhydrique *N*: 1 vol.). L'élution peut être obtenue également par l'éthanol à 80% contenant 0.3% d'ammoniaque (BAILEY ET BETTELHEIM[5]). L'éluat peut être évaporé. Si le résidu est traité par le fluorodinitrobenzène, l'ε-DNP-lysine devient de la di-DNP-lysine, l'O-DNP-tyrosine de la di-DNP-tyrosine, l'imidazole-DNP-histidine de la di-DNP-histidine, tandis que l'α-mono-DNP-arginine n'est pas modifiée. Après cette seconde dinitrophénylation, l'extrait éthéré du résidu enlève la di-DNP-lysine et la di-DNP-tyrosine, une extraction à l'acétate d'éthyle peut enlever la di-DNP-histidine, tandis que l'α-mono-DNP-arginine reste dans la phase aqueuse (voir ROVERY, FABRE ET DESNUELLE[75]).

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* Il est indispensable d'éliminer les fines particules de talc qui gênent l'écoulement. Par ébullition de la suspension de talc dans l'acide chlorhydrique 0.01 *N*, les fines particules se rassemblent à la surface du liquide et peuvent être enlevées facilement. Le lavage est répété plusieurs fois de suite (BAILEY ET BETTELHEIM[5]).

2. *Extraction d'un hydrolysat partiel de protéine*

Les modalités d'extraction sont identiques à celles des hydrolysats totaux. Des extractions successives à l'éther, à l'acétate d'éthyle et au *n*-butanol (WOOLLEY[94], SANGER[78]) peuvent réaliser un certain fractionnement de base du mélange.

Il est très important de pouvoir séparer aussi complètement que possible les α-DNP-peptides de la séquence N-terminale dont la forme acide est, en principe, soluble dans les solvants organiques, et les peptides non terminaux colorés en jaune et contenant de l'ε-DNP-lysine qui sont, en principe, retenus dans la phase aqueuse par leur groupement aminé libre. Cette séparation n'est malheureusement pas toujours très nette (DESNUELLE ET FABRE[23], SCHROEDER[82]).

Les extraits à l'acétate d'éthyle peuvent contenir de l'ε-mono-DNP-lysine. Il est utile de laver les diverses phases organiques plusieurs fois à l'eau et d'étudier la composition de ces liquides de lavage.

Les peptides contenant de l'ε-DNP-lysine peuvent être séparés des autres peptides non dinitrophénylés et des acides aminés libres sur une colonne (1 cm de diamètre) contenant 5 g de talc lavé à l'acide chlorhydrique *N* (SANGER[78]). Les peptides contenant de l'ε-DNP-lysine sont fixés sur la colonne. Après lavage par l'acide chlorhydrique *N* (40 ml), leur élution est obtenue par un mélange d'éthanol (4 parties) et d'acide chlorhydrique (1 partie) ou, de préférence, par de l'éthanol à 80% contenant 0.3% d'ammoniaque.

E. DINITROPHÉNYLATION D'UN PEPTIDE

La dinitrophénylation d'un peptide peut se conduire en pratique exactement de la même façon que celle d'une protéine. Cependant, quelques méthodes (des microméthodes surtout) sont spécialement adaptées à cette réaction de condensation. Les quantités de peptides employées sont généralement très petites.

1. *Condensation en milieu triméthylamine* (SANGER ET THOMPSON[79])

Le remplacement du bicarbonate de sodium par la triméthylamine permet la diminution de l'ionisation du milieu; d'autre part, ce dernier réactif est commodément éliminé par la suite. Le peptide (0.2 µmole par exemple) est dissous dans 0.1 ml de triméthylamine à 1%. On ajoute une solution de 10 µl de fluorodinitrobenzène dans 0.2 ml d'éthanol. Après 2 heures de contact, quelques gouttes d'eau et de la solution de triméthylamine sont ajoutées et l'excès de fluorodinitrobenzène est extrait 3 fois à l'éther ou à l'éther contenant 1% de triéthylamine (HAUSMANN *et al.*[32]). Après évaporation à sec de la solution aqueuse, le résidu est hydrolysé directement par de l'acide chlorhydrique 5.7 *N*.

2. *Condensation en milieu carbonate de triméthylamine*

Pour réduire la formation de dinitrophénol, LOCKHART ET ABRAHAM[50] remplacent la triméthylamine par le carbonate de triméthylamine d'après le mode opératoire suivant.

Le peptide (50–150 µg) est dissous dans 0.1 ml d'une solution à 1.5% de carbonate de triméthylamine (poids/vol.) (pH 9.3). On ajoute 0.01 ml de fluorodinitrobenzène dans 0.2 ml d'éthanol et la réaction se poursuit à l'obscurité pendant 2 h 30 min. L'éthanol est chassé sous pression réduite et on ajoute 0.24 ml de solution de carbonate de triméthylamine. L'excès de fluorodinitrobenzène est extrait à l'éther et la solution aqueuse est évaporée à siccité sous vide. Le résidu, dissous dans 0.1 ml d'acide chlorhydrique 6 *N*, est hydrolysé pendant 9 h à 105° dans un tube scellé sous azote. L'hydrolysat est dilué avec 2 vol. d'eau et les DNP-aminoacides éthérosolubles sont extraits 3 fois avec un volume égal d'éther sulfurique. La di-DNP-histidine, si elle est présente, peut être extraite avec le *n*-butanol ou l'acétate d'éthyle.

WALEY[91] préconise une méthode assez voisine de la précédente avec un tampon carbonate de triméthylamine obtenu par traitement d'une solution à 6% (vol./vol.) de triméthylamine par du gaz carbonique jusqu'à alcalinité au rouge de phénol, mais neutralité à la phénolphtaléine. Dans ces conditions, la réaction de dinitrophénylation est conduite à un pH un peu plus bas que dans la méthode de LOCKHART ET ABRAHAM. Il se forme un peu de dinitrophénol.

F. CHROMATOGRAPHIE SUR PAPIER DES DÉRIVÉS DINITROPHÉNYLÉS ÉTHÉROSOLUBLES

La séparation de tous les dérivés dinitrophénylés éthérosolubles doit se faire à l'aide d'une chromatographie bidimensionnelle.

Trois types de chromatogramme peuvent être employés. Exceptionnellement, dans les cas de mélanges très simples, on peut se contenter d'une (ou plusieurs) chromatographies unidimensionnelles, à condition de se repérer par rapport à des témoins latéraux de DNP-aminoacides.

a. Solvants "toluène" et "phosphate" (BISERTE ET OSTEUX[11], LEVY[43])

La cuve pour la première dimension ascendante de la chromatographie est constituée par un récipient en verre, cylindrique ou parallélépipédique (hauteur: 46 cm, base de 23 × 23 cm) qui doit être très hermétiquement fermé par une plaque de verre bien plate. L'étanchéité de la cuve est assurée par un dépôt de lubrifiant à base de silicone sur les bords de la cuve ou mieux par un joint de caoutchouc mousse placé entre les bords de la cuve et le couvercle. Un poids assure le maintien en place de ce couvercle. Dans le fond de la cuve, on dispose l'un dans l'autre deux cristallisoirs à fond bien plat (diamètres 19 cm et 15 cm) qui laissent subsister entre eux un espace annulaire important. L'ensemble est placé à l'obscurité dans une chambre à température constante (21° ± 0.5°).

La première dimension de la chromatographie est réalisée avec le système "toluène" de BISERTE ET OSTEUX[11] modifié par LEVY[43], dont la composition est la suivante: toluène–pyridine–monochlorhydrine du glycol (ou 2-chloroéthanol)–ammoniaque 0.8 *N* (30:9:18:18). Les différents réactifs doivent être très soigneusement purifiés*.

Le solvant "toluène", qui est biphasique, est équilibré après une seule agitation de l'ampoule au moins pendant 3 à 4 h. La phase aqueuse inférieure est rejetée et la phase organique est filtrée sur du papier Whatman No. 1 pour enlever les quelques gouttelettes d'eau qui restent.

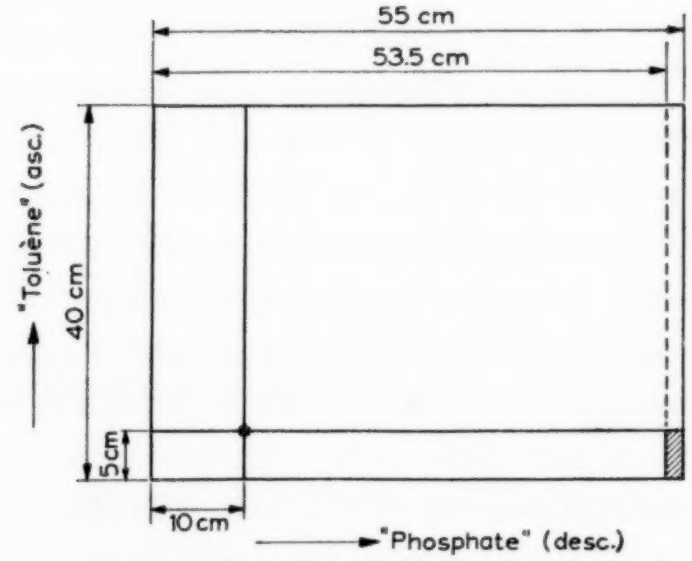

Fig. 4. Disposition du chromatogramme (papier Whatman No. 1). desc. = chromatographie descendante; asc. = chromatographie ascendante. Après avoir roulé en cylindre la feuille de papier et agrafé les deux bords, il est recommandé de découper une surface de papier (surface hachurée de la figure; 5 × 1.5 cm) afin d'éviter une montée trop rapide du solvant "toluène" par capillarité.

Les dérivés dinitrophénylés en solution acétonique sont déposés à l'aide d'une micropipette dans le coin d'une feuille de papier Whatman No. 1 (40 × 55 cm) (voir Fig. 4). Après le dépôt des DNP-aminoacides, la feuille de papier est roulée en cylindre (la forme cylindrique est maintenue par des agrafes).

Le rouleau est posé dans l'espace annulaire compris entre les deux cristallisoirs. On place dans le cristallisoir central 200 ml d'ammoniaque 0.8 *N* (57 ml d'ammoniaque concentrée à 22° Bé, densité = 0.925, dans 1 l d'eau) et, directement dans le fond de la cuve, en dehors des cristallisoirs, 30 à 40 ml de la phase organique du solvant "toluène" préparé comme il est indiqué ci-dessus. La cuve à chromatographie est très soigneusement fermée et on laisse s'équilibrer le papier avec l'atmosphère de la cuve au minimum pendant 5 à 6 h. La prolongation de ce temps d'équilibration (24 h par exemple) améliore encore la séparation. Il faut signaler que des chromatogrammes plus satisfaisants sont obtenus dans des cuves en état d'utilisation continue.

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* *Toluène*: une extraction par de l'acide sulfurique pur (100 ml d'acide par l de toluène) pendant 24 h; après décantation, distillation sur chlorure d'aluminium, lavage au carbonate de sodium à 20%, lavage à l'eau à plusieurs reprises (5 fois), filtration sur un filtre contenant des morceaux de papier qui servent à fixer l'eau, conservation sur du chlorure de calcium sec et redistillation; *chloroéthanol*: distillation; *pyridine*: distillation sur baryte et redistillation.

Au bout de ce temps, on verse rapidement dans l'espace annulaire et à l'aide d'un entonnoir à longue tige, une quantité suffisante (50 ml) de phase organique du solvant "toluène". La chromatographie ascendante dure 15 h. Le lendemain, les feuilles sont enlevées, séchées par ventilation d'air tiède pendant 10 à 12 h *à l'abri de la lumière* sous une sorbonne. L'enlèvement du solvant de la première dimension doit être complet.

La feuille est alors dépliée, retournée et placée dans une cuve en verre (dimensions: hauteur 48 cm, base 46 × 36 cm) bien étanche, pour une chromatographie descendante avec un système solvant constitué par un tampon phosphate 1.5 M de pH 6: PO_4H_2Na 1 M, PO_4HNa_2 0.5 M, soit 138 g de $PO_4H_2Na \cdot H_2O$ et 71 g de PO_4HNa_2 par l, ou 156 g de $PO_4H_2Na \cdot 2H_2O$ et 89 g de $PO_4HNa_2 \cdot 2H_2O$ (sel de Sørensen) par l*.

La chromatographie peut être mise en route immédiatement sans temps d'équilibration. Il est nécessaire de placer dans le fond de la cuve un godet rempli d'eau. La durée de la chromatographie est de 15 h dans une salle à température constante (+ 21° ± 0.5°) et à l'obscurité. Le chromatogramme est séché pendant 6 h au moins par ventilation d'air tiède dans une sorbonne obscure.

Ce type de chromatographie bidimensionnelle permet de séparer la plupart des DNP-aminoacides éthérosolubles, sauf les DNP-aminoacides dicarboxyliques (acide DNP-aspartique, acide DNP-glutamique). Ces deux dérivés peuvent être séparés en effectuant un second chromatogramme, en première dimension avec le système "toluène", en seconde dimension avec du tampon phosphate de concentration plus forte (2.5 M), la chromatographie descendante étant prolongée pendant 48 h. La séparation obtenue dans le cas d'un mélange de DNP-aminoacides éthérosolubles d'un hydrolysat total de protéine est schématisée dans la Fig. 5.

Il n'est pas nécessaire de définir la position de chaque tache par des R_F dans les deux dimensions. En effet, la position relative de chaque dérivé est toujours très caractéristique, et, de plus, les valeurs de R_F, notamment dans le solvant "toluène", peuvent varier, dans une certaine limite, suivant le type de cuve à chromatographie. Parmi les autres causes de variations des R_F, on peut encore citer le degré de saturation de l'atmosphère de la cuve, le vieillissement du solvant "toluène", la distance parcourue par le front du solvant, la composition du mélange et la quantité de DNP-aminoacides. Dans le cas de mélanges simples, dans lesquels plusieurs DNP-aminoacides sont absents, l'identification exacte se fait aisément et sûrement en plaçant sur le cercle de départ, en même temps que le mélange à étudier, un certain nombre de témoins-internes qui peuvent venir "encadrer" les taches d'identification délicate. Les traces de dinitrophénol qui peuvent encore se trouver dans les mélanges à étudier et qui se décolorent facilement et entièrement au contact de vapeurs d'acide formique, servent également de repère pour l'identification des DNP-aminoacides. Le repérage et la localisation de faibles quantités de DNP-aminoacides sont facilités par un examen *rapide* en lumière de Wood (fluorescence brune); la 2,4-dinitraniline, qui est un artefact constant de la méthode, présente dans ces conditions une fluorescence très particulière de teinte jaune vert. Elle possède d'ailleurs un R_F voisin de 1 dans le système "toluène" et très faible dans le tampon phosphate.

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* Il est recommandé de préparer un tampon de molarité plus élevée: 3 M (PO_4H_2Na 2 M PO_4HNa_2 1 M, soit 312 g de $PO_4H_2Na \cdot 2H_2O$ et 178 g de $PO_4HNa_2 \cdot 2H_2O$ (sel de Sørensen) par l). Cette solution peut être diluée au moment de la mise en route du chromatogramme. Elle est conservée à l'étuve à 37°.

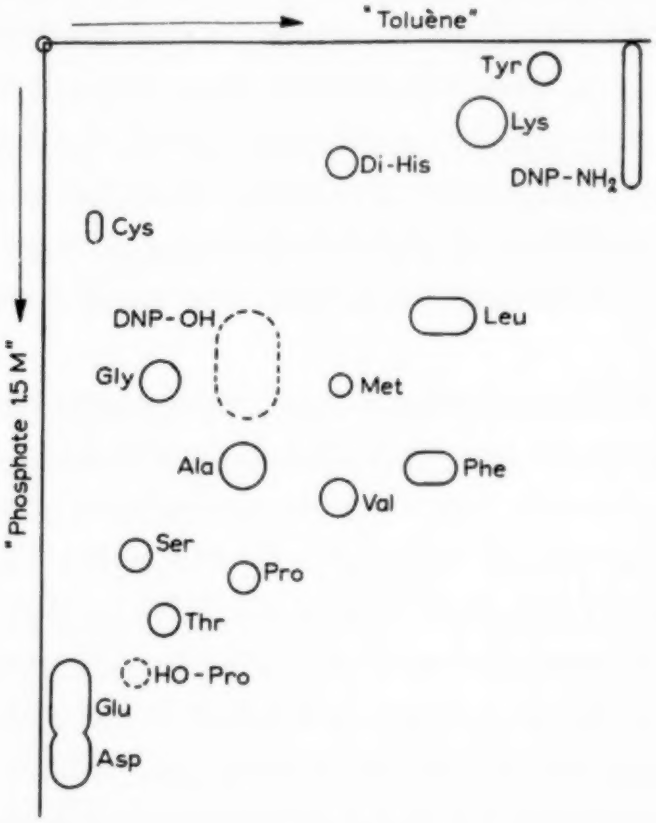

Fig. 5. Carte chromatographique des DNP-aminoacides éthérosolubles d'un hydrolysat total de protéine. Systèmes solvants "toluène" et "phosphate 1.5 *M*". DNP-NH$_2$ = dinitraniline; DNP-OH = dinitrophénol. Sur le schéma, les dinitrophénylaminoacides sont notés comme les acides aminés correspondants, ex.: Ala = DNP-alanine. HO-Pro = DNP-hydroxyproline; Di-His = di-DNP-histidine.

b. Solvants "n-butanol-NH$_3$" et "phosphate"

Braunitzer[15] a préconisé l'emploi en première dimension ascendante du système *n*-butanol saturé par de l'ammoniaque à 0.1% (poids/vol.) (voir aussi Koch et Weidel[39], Davies et Harris[22]). On peut également employer le système propanol–ammoniaque à 0.2% (poids/vol.) (75:25). Il est préférable de réaliser la chromatographie sur du papier Schleicher et Schüll No. 2043a ou 2043b.

La seconde dimension est faite soit avec le tampon phosphate 1.5 *M* (pH 6) de Levy (Koch et Weidel[39]), soit avec un tampon phosphate plus dilué, 0.75 *M* de pH 6 (Davies et Harris[22]). La durée de la chromatographie avec le phosphate 0.75 *M* est réduite à 7–8 heures.

Les modalités expérimentales pour la conduite de la chromatographie sont en tous points comparables à celles que nous avons décrites pour la chromatographie "toluène" et "phosphate". La position des DNP-aminoacides éthérosolubles est schématisée dans la Fig. 6.

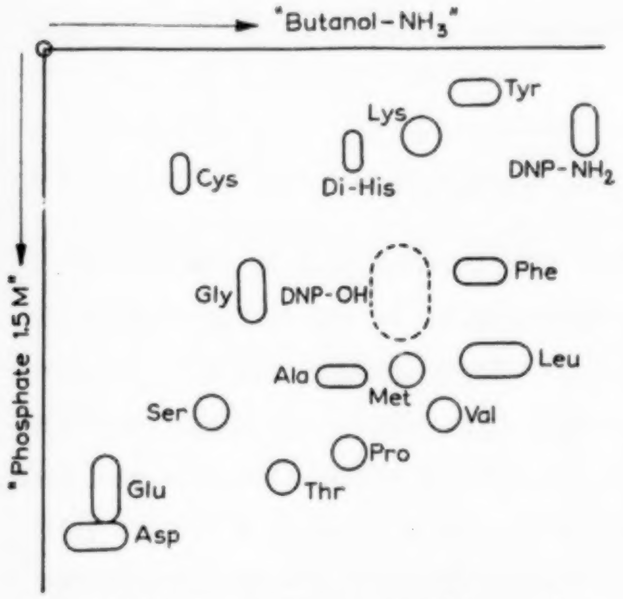

Fig. 6. Carte chromatographique des DNP-aminoacides éthérosolubles d'un hydrolysat total de protéine. Systèmes solvants "butanol–NH$_3$" et "phosphate 1.5 M". DNP-NH$_2$ = dinitraniline; DNP-OH = dinitrophénol; Di-His = di-DNP-histidine.

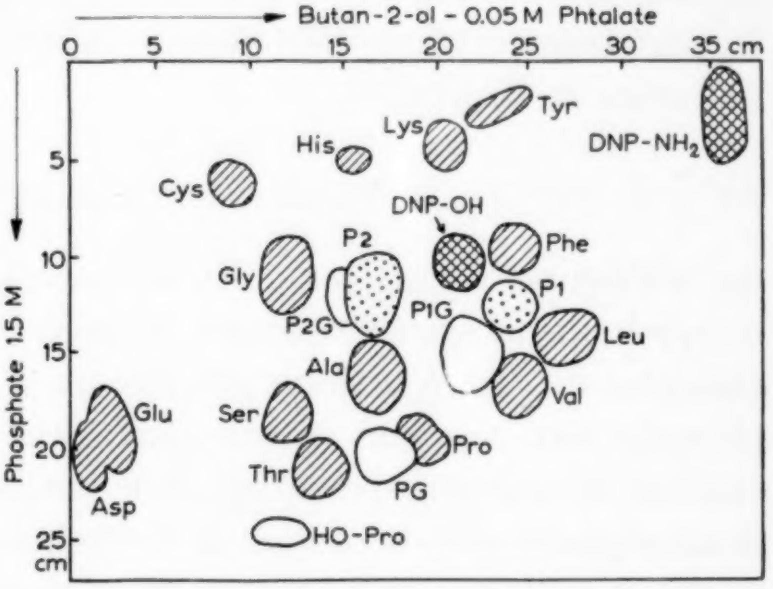

Fig. 7. Carte chromatographique des DNP-aminoacides éthérosolubles d'un hydrolysat total de protéine. Systèmes solvants "*sec.*-butanol–phtalate pH 6" et "phosphate 1.5 M" (d'après Phillips[64]). DNP-OH = dinitrophénol; DNP-NH$_2$ = dinitraniline; P1 = acide δ-chloro-α-DNP-aminovalérique; P2 = acide α-chloro-δ-DNP-aminovalérique; PG = DNP-prolylglycine; P1G = δ-chloro-α-DNP-aminovalérylglycine; P2G = probablement α-chloro-δ-DNP-aminovalérylglycine. Les DNP-aminoacides éthérosolubles habituellement rencontrés dans un hydrolysat sont indiqués par des surfaces hachurées. Les produits de décomposition de la DNP-proline sont figurés par des surfaces pointillées. L'ε-DNP-lysine (hydrosoluble) coïncide avec la DNP-sérine.

c. Solvants "sec.-butanol–phtalate pH 6" et "phosphate"

Phillips[64] a décrit une chromatographie bidimensionnelle sur papier Whatman No. 7 dans laquelle la première dimension s'effectue avec le solvant butan-2-ol saturé avec le tampon phtalate 0.05 M de pH 6* (durée de développement 15 à 20 h). Le papier est, au préalable, tamponné avec le tampon phtalate 0.05 M. La seconde dimension est réalisée avec le système tampon phosphate 1.5 M de pH 6 de Levy (durée de développement: une nuit) (Fig. 7).

d. Solvants "alcool tert.-amylique–phtalate pH 6" et "phosphate 1.5 M"

Phillips[64] a également proposé un couplage bidimensionnel constitué en première dimension ascendante par le système de Blackburn et Lowther[13] (alcool *tert.*-amylique—ou 2-méthylbutan-2-ol—saturé avec le tampon phtalate 0.05 M de pH 6*; durée de développement dans l'obscurité: 30–40 h). Une équilibration prolongée

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* *Formule du tampon phtalate*: 50 ml de diphtalate de potassium 0.1 M (20.418 g par l) et 45.45 ml de NaOH 0.1 N; diluer à 100 ml avec de l'eau privée de CO$_2$.

(une nuit) de la feuille avant le départ de la chromatographie améliore les résultats (INGRAM ET SALTON[36]). La seconde dimension est réalisée avec le tampon phosphate 1.5 *M* de pH 6 de LEVY, après un séchage à la température du laboratoire.

Avant la première dimension, les feuilles de papier Whatman No. 7 sont tamponnées avec le tampon phtalate 0.05 *M* de pH 6. Le papier Whatman No. 4 ou 3 MM est parfois employé avec le système solvant de BLACKBURN ET LOWTHER (LOCKHART, ABRAHAM ET NEWTON[51]).

e. Valeurs comparées des différentes techniques

Ces différentes techniques diffèrent essentiellement entre elles par la nature et la composition du système solvant de la première dimension. Il est remarquable de constater que la répartition générale et les positions relatives des dérivés dinitrophénylés sont toujours sensiblement les mêmes, quel que soit le système solvant employé en première dimension. Il ne semble donc pas que la séparation réalisée soit la conséquence directe des lois de la chromatographie de partage pure. D'autre part, la chromatographie en seconde dimension consiste essentiellement en un "relargage" tout le long de la feuille de papier.

Suivant les couplages réalisés, la position du dinitrophénol, qui est le principal artefact de la méthode de SANGER[76], peut varier d'une façon importante. Dans le couplage "toluène + phosphate", le dinitrophénol est voisin du DNP-glycocolle et de la DNP-alanine. Dans les couplages "*n*-butanol–NH_3 + phosphate" et "*sec.*-butanol–phtalate pH 6 + phosphate", le dinitrophénol est beaucoup plus près de l'ensemble DNP-phénylalanine, DNP-leucine, DNP-valine, DNP-méthionine, et cette position est beaucoup plus gênante. Ces inconvénients sont naturellement réduits si le dinitrophénol a été éliminé par sublimation.

Néanmoins, en définitive, nous pensons que la résolution la plus satisfaisante est obtenue par le système "toluène + phosphate 1.5 *M*". L'utilisation d'un tampon phosphate 0.75 *M* permet de réduire la durée de la seconde dimension. Il est certain, toutefois, comme le signale PHILLIPS[64], qu'il y a certains inconvénients à employer le système "toluène–pyridine–chloroéthanol–ammoniaque" dans les laboratoires où l'on effectue des dosages d'acides aminés par les méthodes colorimétriques à la ninhydrine.

f. Autres systèmes solvants

Tous les problèmes posés par la séparation des DNP-aminoacides éthérosolubles peuvent être résolus par les couplages bidimensionnels décrits ci-dessus. Quelques problèmes simples peuvent être abordés par l'emploi de l'un des systèmes solvants précédents en chromatographie unidimensionnelle, à condition de placer sur le chromatogramme des substances-témoins de référence (témoins latéraux et témoins internes). Toutefois, d'autres systèmes solvants ont été proposés. Leur emploi est cependant limité à quelques problèmes très particuliers. Parmi ceux qui sont les plus utiles, citons:

Eau–benzène–acide acétique (1:1:1) (PARTRIDGE ET DAVIS, cité dans SANGER ET

Bibliographie p. 269/271.

THOMPSON[79]), utilisable pour les DNP-aminoacides de R_F faible dans les solvants organiques: acide DNP-aspartique, acide DNP-glutamique, DNP-sérine et éventuellement acide DNP-cystéique.

Systèmes solvants de MELLON *et coll.*[55] (voir Tableau IV):

Solvant A = *n*-butanol saturé d'eau;

Solvant B = *n*-butanol–acétate de *n*-butyle–ammoniaque à 1% (v/v) (1:2:3) (préparé 18 h avant l'usage);

Solvant C = benzène–acide acétique à 1%; la chromatographie est ascendante, sur papier Whatman No. 1; le papier est équilibré une nuit avant la mise en place du solvant organique; le système solvant C donne des traînées.

TABLEAU IV

VALEURS DES R_F DES 2,4-DINITROPHÉNYLAMINOACIDES
(D'après MELLON, KORN ET HOOVER[55])

Acides aminés	*Solvant A**	*Solvant B*	*Solvant C*
Dinitroaniline	0.90	0.97	0.96
Di-DNP-tyrosine	0.78	0.90	0.33
Leucine	0.74	0.71	0.70
Isoleucine	0.73	0.70	0.70
Di-DNP-lysine	0.72	0.81	0.11
Phénylalanine	0.71	0.70	0.55
Tryptophanne	0.70	0.68	0.28
Valine	0.68	0.47	0.63
Méthionine	0.65	0.48	0.47
Dinitrophénol	0.56	0.25	0.99
Alanine	0.50	0.18	0.28
Proline	0.48	0.17	0.44
Thréonine	0.43	0.12	0
Glycocolle	0.36	0.08	0.07
Sérine	0.32	0.06	0
Acide glutamique	0.14	0	0
Acide aspartique	0.12	0	0
Di-DNP-histidine	0.35	0.50	0
Arginine	0.37	0	0
ε-DNP-lysine	0.32	0.05	0
α-DNP-lysine	0.33	0	0

* Pour la composition des solvants, voir le texte.

Système isooctane–monochlorhydrine du glycol–n-propanol (20:1:1) (WILLIAMSON ET PASSMANN[92]): Séparation de la DNP-leucine et de la DNP-phénylalanine.

Système décaline (décahydronaphtalène)–acide acétique pur cristallisable (1:1) (BISERTE ET OSTEUX[11]), utilisable pour la séparation chromatographique du dinitrophénol et des DNP-aminoacides.

n-Propanol (dilué avec de l'eau pour obtenir une densité de 0.813)–acide acétique (contenant 1.5% d'eau)-kérosène (E: 100–140°) (20:6:100), en chromatographie descendante sur Whatman No. 1 trempé dans l'acide citrique 0.1 *M* et séché à l'air; utilisable pour la séparation des polymères d'α-, β- et ω-aminoacides (HEIKENS, HERMANS ET VAN VELDEN[33]).

Xylène–acide acétique–tampon phtalate 0.05 M de pH 6 (10:5:4) (LANDMANN, DRAKE ET WHITE[42]); le papier est tamponné avec le même tampon, puis équilibré avec la couche inférieure 16 h avant la mise en route.

n-Butanol–éthanol–eau (40:10:50) (v/v) (KENT, LAWSON ET SENIOR[38]).

Alcool benzylique additionné de 10% d'éthanol (v/v) et saturé avec le tampon phtalate 0.05 M de pH 6 (BLACKBURN ET LOWTHER[13]) (voir Tableau V).

Propanol–cyclohexane ou éther de pétrole (E: 100–120°) (30:70 v/v), saturé avec le tampon phtalate 0.05 *M* de pH 6 (BLACKBURN ET LOWTHER[13]) (voir Tableau V).

TABLEAU V

R_F DES DNP-AMINOACIDES SUR PAPIER TAMPONNÉ (TAMPON PHTALATE DE pH 6)
(D'après BLACKBURN ET LOWTHER[13])

	Cyclohexane contenant 30% de propanol	*Alcool tert.-amylique*	*Alcool benzylique contenant 10% d'éthanol*
DNP-leucine	0.28	0.88	0.71
DNP-valine	0.23	0.79	0.59
DNP-phénylalanine	0.22	0.74	0.63
DNP-alanine	0.15	0.46	0.36
DNP-glycocolle	0.10	0.23	0.26
DNP-thréonine	0.07	0.36	0.26
DNP-sérine	0.05	0.21	0.18
Acide DNP-glutamique	0.05	0.04	0.07
Acide DNP-aspartique	0.02	lent	0.03

Tampon citrate de sodium–acide chlorhydrique M ou 0.7 M de pH 6.2 (ROVERY ET FABRE[74], DESNUELLE ET FABRE[23]); durée: 16 h; c'est en fait une application de la chromatographie dite "de relargage", comparable à celle réalisée avec le tampon phosphate 1.5 *M* de pH 6 de LEVY.

Solvant de BLACKBURN ET LOWTHER *modifié*: *tert.-pentanol contenant 10% (v/v) de propan-2-ol saturé avec du tampon phtalate* (GREGORY ET YOUNG, résultats non publiés, cités dans WALEY[91]).

Chloroforme–acide acétique 1.5 N–n-propanol (10:6:10) (SANGER ET THOMPSON[79]): chromatographie en phase inversée sur du papier siliconé (KRITCHEVSKY ET TISELIUS[41]); le papier est suspendu dans la cuve saturée de vapeurs de la phase organique pendant 3 h; puis le développement se fait avec la phase aqueuse; la di-DNP-tyrosine et la di-DNP-lysine ne migrent pas, tandis que les autres DNP-aminoacides migrent assez rapidement. A cause de la variabilité des R_F, il est indispensable de mettre des témoins latéraux de DNP-aminoacides.

Chloroforme–propan-2-ol–benzoate de potassium 0.05 M (45:49:6 v/v) ou *cyclohexane–propan-2-ol–benzoate de potassium 0.05 M (60:36:4 v/v)* (MONIER ET PENASSE[60]).

Solvant alcool n-amylique agité avec un volume égal de solution d'ammoniaque 2 N; la phase aqueuse est utilisée pour la saturation de la cuve (BOWES ET MOSS[14]).

Alcool tert.-amylique–méthyléthycétone–benzoate de potassium (54:40:6) (MONIER ET JUTISZ[59]).

G. MODALITÉS D'APPLICATION PARTICULIÈRES DE LA CHROMATOGRAPHIE DES DNP-AMINOACIDES ÉTHÉROSOLUBLES

a. Hydrolysats totaux d'une DNP-protéine et DNP-aminoacides d'un hydrolysat total

Tous les problèmes peuvent être abordés par les couplages bidimensionnels décrits ci-dessus. Nous donnons cependant la préférence aux systèmes "toluène" et "phosphate 1.5 M". Quelques cas particuliers sont très délicats à résoudre. Il est parfois nécessaire d'éluer les taches de DNP-aminoacides du chromatogramme afin de pouvoir les étudier de nouveau avec d'autres systèmes solvants.

1. *Élution des taches de DNP-aminoacides*

L'élution du papier est conduite de la façon suivante. Les zones de papier contenant les DNP-aminoacides sont découpées, placées dans des tubes à centrifuger et éluées par 2 à 3 ml de bicarbonate de sodium à 2% pendant 15 min à une température de 50-55°. Après refroidissement et acidification du milieu par de l'acide chlorhydrique dilué, les DNP-aminoacides sont extraits par l'éther sulfurique privé de peroxydes. Afin d'éliminer des liqueurs éthérées toute trace de solvant employé au cours de la première chromatographie, celles-ci sont de nouveau soumises à une extraction par 2 à 3 ml de bicarbonate de sodium à 2% et, après acidification, la seconde solution bicarbonatée est extraite à l'éther. Cette solution éthérée (2 à 3 ml au maximum) peut être déshydratée sur du sulfate de sodium anhydre. Après évaporation ou concentration, les DNP-aminoacides sont alors déposés sur la feuille de papier Whatman No. 1 et soumis à une chromatographie unidimensionnelle dans le nouveau système solvant.

2. *Séparation des acides DNP-dicarboxyliques*

Seuls, les acides DNP-aspartique et DNP-glutamique ne sont pas séparés par les couplages bidimensionnels. Ce problème peut être résolu par une seconde chromatographie bidimensionnelle dans laquelle la deuxième dimension est réalisée avec un tampon phosphate de concentration plus élevée (2.5 M), la première dimension pouvant se faire cette fois de préférence avec le système butanol (NH_3) qui s'évapore plus rapidement que le système "toluène".

On peut aussi éluer du chromatogramme bidimensionnel les taches des DNP-aminoacides dicarboxyliques et soumettre l'éluat à une chromatographie unidimensionnelle dans le système alcool isoamylique saturé d'acide acétique à 1% (R_F de l'acide DNP-aspartique 0.30; R_F de l'acide DNP-glutamique 0.46) (BISERTE ET OSTEUX[11]).

3. *Superpositions des taches de DNP-aminoacides*

(*i*) *Superposition DNP-histidine–DNP-tryptophanne.* Dans le couplage bidimensionnel "toluène + phosphate" la DNP-histidine et le DNP-tryptophanne se placent au même endroit. Cette concordance ne pose toutefois pas de problèmes particuliers*, car, d'une part, le DNP-tryptophanne est détruit au cours de l'hydrolyse acide et d'autre part, la di-DNP-histidine n'accompagne les DNP-aminoacides éthérosolubles que dans le cas d'une extraction couplée éther + acétate d'éthyle.

(*ii*) *Superposition DNP-sérine–DNP-méthionine-sulfone.* Dans le système "toluène–phosphate 1.5 *M*", la DNP-sérine et la DNP-méthionine-sulfone se superposent. En fait, la formation de la DNP-méthionine-sulfone aux dépens de la DNP-méthionine n'a lieu qu'au cours de l'extraction des DNP-aminoacides par de l'éther qui n'a pas été privé de peroxydes. Si la présence de DNP-méthionine est soupçonnée, la zone DNP-sérine + DNP-thréonine peut être chromatographiée de nouveau dans le système alcool *tert.*-amylique–phtalate de BLACKBURN ET LOWTHER[13].

(*iii*) *Dérivés dinitrophénylés des acides diaminés.* La di-DNP-lysine, la di-DNP-ornithine et l'acide di-DNP-diaminobutyrique ne se séparent pas dans le couplage "toluène–phosphate 1.5 *M*". Une séparation partielle de la di-DNP-lysine et de la di-DNP-ornithine peut être obtenue en chromatographie avec le système butanol–acide acétique–eau (4:1:5).

L'identification de ces composés est donc délicate. Pour résoudre ce problème (qui peut se poser parfois dans le domaine des polypeptides bactériens), il est donc indispensable de régénérer les acides aminés constitutifs (voir plus loin, paragraphe G, a, 4) et d'identifier ceux-ci, par exemple par une électrophorèse sur papier dans un appareil "en toit" (type Durrum) à pH 3.9 [tampon pyridine–acide acétique–eau (30:100:4870)] pour la séparation de l'acide α,γ-diaminobutyrique du groupe ornithine + lysine, ou à pH 11.7 (ammoniaque *N*) pour la séparation de la lysine et de l'ornithine.

4. *Problème des DNP-leucines*

Aucun système solvant ne permet une séparation satisfaisante des DNP-leucines. Au cours de la détermination d'un résidu en postion N-terminale, le seul moyen de résoudre la question consiste à régénérer l'acide aminé à partir de son dérivé dinitrophénylé.

La régénération** peut se faire par chauffage dans un tube scellé à 105° pendant 1 h avec de la baryte 0.3 *N* (MILLS[58]). Ensuite, on élimine la baryte sous forme de carbonate de baryum en faisant passer dans la solution un courant de gaz carbonique. Après évaporation à sec, le résidu est analysé en chromatographie sur papier.

On peut également chauffer les DNP-aminoacides en présence d'ammoniaque (densité 0.880) en tubes scellés à 100° (LOWTHER[52]).

La séparation chromatographique de la leucine et de l'isoleucine peut être

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[*] La séparation du DNP-tryptophanne et de la di-DNP-histidine peut être obtenue dans les systèmes de MELLON: benzène–acide acétique à 1%; *n*-butanol saturé d'eau (voir Tableau IV), et dans le système *sec.*-butanol–tampon phtalate de pH 6 (R_F du DNP-Try = 0.54; R_F de la DNP-His = 0.33). Durée de la chromatographie: 40 h (résultats personnels).

[**] Ces méthodes de régénération sont valables pour tous les DNP-aminoacides; elles sont utilisables uniquement pour une identification, car les rendements en acides aminés régénérés sont faibles.

obtenue soit dans le système *tert.*-pentanol–eau (Work[95]), soit dans le système de Högström[35], soit dans le mélange *n*-butanol–alcool benzylique–eau (Consden *et al.*[20]).

5. *Produits de décomposition de la DNP-proline*

Au cours de l'hydrolyse de la DNP-proline, deux dérivés nouveaux peuvent se former: l'acide δ-chloro-α-DNP-aminovalérique et l'acide α-chloro-δ-DNP-aminovalérique (Scanes et Tozer[81])*. La destruction de la DNP-proline et l'apparition de ces composés dépendent des conditions d'hydrolyse (voir paragraphe D, c, 1, p. 239).

Il est d'ailleurs commode d'identifier la DNP-proline en position N-terminale par l'intermédiaire de ces deux artefacts (Phillips[64]). Leur séparation chromatographique soit dans le couplage "toluène" et "phosphate" (voir Fig. 8) soit dans le couplage "*sec.*-butanol–phtalate" et "phosphate" (voir Fig. 7) est relativement commode. Il est intéressant de signaler que la DNP-proline prend une coloration rouge après exposition prolongée du chromatogramme à la lumière de Wood.

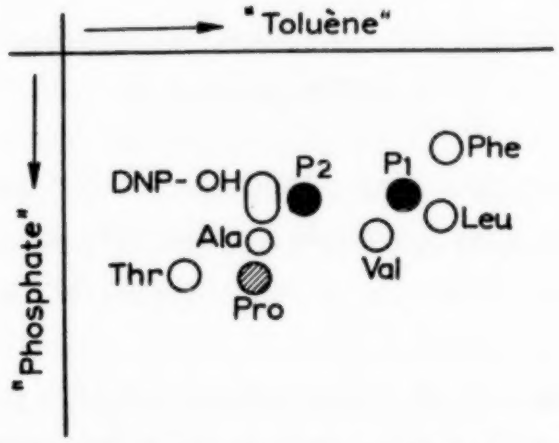

Fig. 8. Étude chromatographique (systèmes solvants "toluène" et "phosphate") des produits de décomposition de la DNP-proline (hydrolyse par l'acide chlorhydrique 5.6 *N* à 100–105° pendant 24 h en tube scellé). DNP-proline = surface hachurée; P1 = acide δ-chloro-α-DNP-aminovalérique P2 = acide α-chloro-δ-DNP-aminovalérique; DNP-OH = dinitrophénol.

6. *Artefacts chromatographiques de la phase éthérosoluble*

Le dinitrophénol et la dinitraniline sont des artefacts habituels de la fraction éthérosoluble. Le dinitrophénol peut être facilement éliminé par sublimation** (voir paragraphe C, c, p. 235). La dinitraniline ne gêne pas la séparation chromatographique, car elle se place loin de tous les autres dérivés dinitrophénylés.

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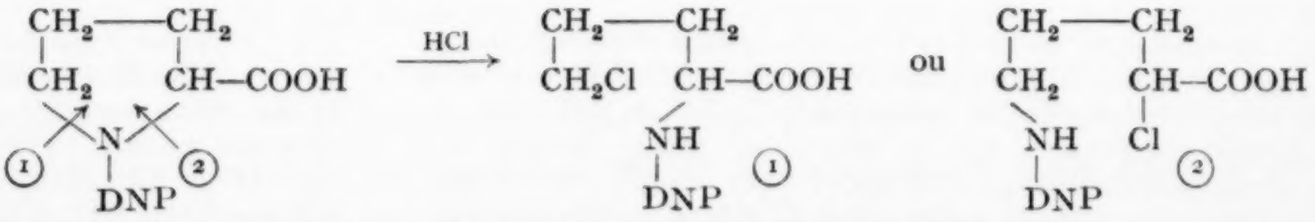
* Sous l'action des agents hydrolysants, il y aurait ouverture du cycle pyrrolidine suivant la réaction:



** Il peut être éliminé également sur colonne d'acide silicique Mallinckrodt, préparé pour la chromatographie par la méthode de Ramsey et Patterson (Li et Ash[48]).

Avec certaines préparations de fluorodinitrobenzène, nous avons parfois constaté la présence d'acide picrique dans une zone voisine de celle qui est occupée par la phénylalanine (voir Fig. 9). L'éluat de la tache donne les réactions caractéristiques et classiques de l'acide picrique.

D'autres artefacts ont également été signalés, notamment par REDFIELD ET ANFINSEN[70]: une tache plus lente que la phénylalanine dans le "toluène" et un peu plus lente que la leucine dans le phosphate; un artefact orangé un peu plus rapide que la glycocolle; un autre artefact orangé dans la région de la valine. Enfin, deux produits de décomposition de l'ε-DNP-lysine (l'un jaune, l'autre orangé) peuvent se placer dans la zone comprise entre la di-DNP-lysine et la di-DNP-histidine. Ils peuvent être confondus avec la di-DNP-histidine.

b. Hydrolysats enzymatiques de protéines; séparation des DNP-asparagine et DNP-glutamine

Le seul problème particulier posé par l'étude des DNP-aminoacides des hydrolysats enzymatiques de protéines (par exemple: action de la carboxypeptidase ou de la leucine-aminopeptidase) est celui de la séparation de la DNP-asparagine et de la DNP-glutamine.

Dans le couplage "toluène" et "phosphate 1.5 *M*", ces deux dérivés forment en effet une tache confluente, mais nettement séparée des autres dérivés dinitrophénylés,

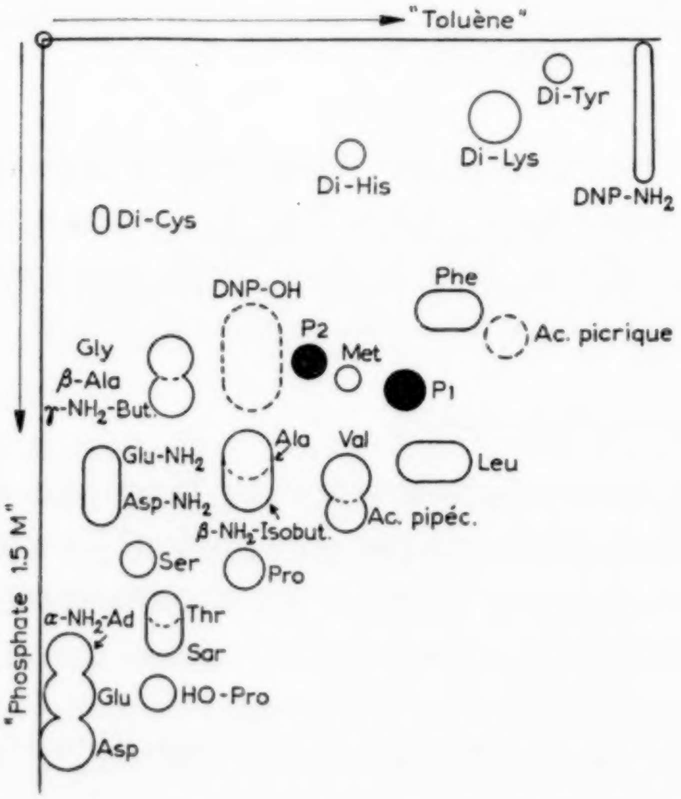

"Toluène"
Di-Tyr
Di-Lys
Di-His
DNP-NH₂
Di-Cys
DNP-OH
Phe
P2
Ac. picrique
Gly
β-Ala
γ-NH₂-But.
Met
P1
Glu-NH₂
Asp-NH₂
Ala
Val
Leu
β-NH₂-Isobut.
Ac. pipéc.
Ser
Pro
α-NH₂-Ad
Thr
Sar
Glu
HO-Pro
Asp
"Phosphate 1.5 M"

Fig. 9. Chromatographie des DNP-aminoacides éthérosolubles décelables après dinitrophénylation d'un milieu biologique. Systèmes solvants "toluène" et "phosphate 1.5 *M*". α-NH_2-Ad. = acide α-aminoadipique; Sar = sarcosine; β-NH_2-Isobut. = acide β-aminoisobutyrique (T-spot); Ac. pipéc. = acide pipécolique; Asp.-NH_2 = asparagine; Glu-NH_2 = glutamine; β-Ala = β-alanine; γ-NH_2-But. = acide γ-aminobutyrique; P1 et P2 = produits de décomposition de la proline (voir Fig. 8).

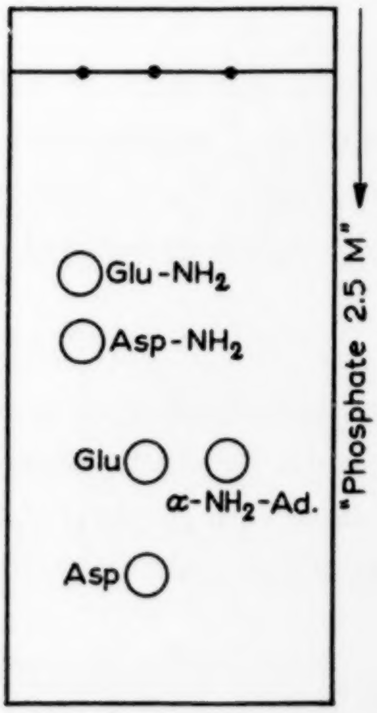

Glu-NH₂
Asp-NH₂
Glu
α-NH₂-Ad.
Asp
"Phosphate 2.5 M"

Fig. 10. Séparation des DNP-aminoacides dicarboxyliques et de leurs amides par le système solvant "phosphate 2.5 *M*". Glu-NH_2 = glutamine; Asp-NH_2 = asparagine; α-NH_2-Ad. = acide α-aminoadipique.

entre le DNP-glycocolle et le groupe DNP-sérine–DNP-thréonine (Fig. 9). Après élution de cette zone, suivant les modalités techniques décrites plus haut (paragraphe G, a, 1, p. 251), la DNP-glutamine et la DNP-asparagine sont facilement séparées en chromatographie unidimensionnelle dans le tampon phosphate 2.5 M (Fig. 10). La glutamine se place derrière l'asparagine.

c. Milieux biologiques complexes

La dinitrophénylation de la fraction "aminoacides" d'un milieu biologique complexe (sang, urine, tissus) conduit à la formation de nombreux dérivés dinitrophénylés éthérosolubles nouveaux, qui viennent le plus souvent se placer au niveau des DNP-aminoacides habituels sur les chromatogrammes "toluène–phosphate 1.5 M" (Fig. 9).

L'acide α-DNP-aminoadipique ne peut être séparé de l'acide DNP-glutamique, même avec un tampon phosphate 2.5 M de pH 6 (Fig. 10).

La DNP-sarcosine se place au niveau de la DNP-thréonine. Elle peut en être distinguée, après élution de leur tache commune, dans le système butanol–acide acétique–eau (4:1:5), l'identification se faisant à l'aide de témoins latéraux: la DNP-sarcosine se place derrière la DNP-thréonine.

La DNP-β-alanine et l'acide γ-DNP-aminobutyrique forment une tache confluente placée sous celle du DNP-glycocolle. Après élution de l'ensemble de la zone, la séparation peut etre faite, en s'aidant de témoins latéraux, en électrophorèse sur papier à pH 3.9 (tampon pyridine–acide acétique–eau (30:100:4870)) sous 300 V pendant 4 h dans un appareil "en toit" (type Durrum). L'ordre croissant de migration vers l'anode est le suivant: acide γ-DNP-aminobutyrique, DNP-β-alanine, DNP-glycocolle. L'utilisation du système *sec.*-butanol–tampon phtalate de pH 6 permet la séparation DNP-glycocolle ($R_F = 0.29$), DNP-alanine ($R_F = 0.43$), DNP-β-alanine ($R_F = 0.52$) (durée de la chromatographie: 40 h) (résultats personnels). Les R_F de l'acide γ-DNP-aminobutyrique dans différents systèmes solvants sont les suivants (KOJIMA *et al.*[40]): n-butanol saturé avec de l'ammoniaque: 0.40; n-butanol–eau–éthanol (4:2:1): 0.90; n-butanol–acide acétique–eau (4:1:5): 0.95; tampon phosphate 0.1 M: 0.66.

L'acide β-DNP-amino-isobutyrique (*T-spot*) coïncide avec la tache de la DNP-alanine. Après élution de cette zone, l'identification de ces deux composés peut se faire, en présence de témoins latéraux, en chromatographie unidimensionnelle dans le butanol–acide acétique–eau (4:1:5). L'alanine se place derrière l'acide β-DNP-amino-isobutyrique.

L'acide DNP-pipécolique ne se sépare pas de la DNP-valine en chromatographie bidimensionnelle. Après élution de cette zone, l'identification de ces composés peut se faire à l'aide de témoins latéraux en électrophorèse sur papier en tampon acide acétique N de pH 2.4 sous 300 V pendant 5 h dans un appareil "en toit" (type Durrum). La DNP-valine migre plus rapidement que l'acide DNP-pipécolique.

Toutes ces identifications sont longues et délicates. Il est indispensable qu'elles soient toujours effectuées en présence des DNP-aminoacides correspondants placés en témoins internes et latéraux.

H. CHROMATOGRAPHIE DES DNP-AMINOACIDES HYDROSOLUBLES

La séparation des DNP-aminoacides hydrosolubles dépend directement des applications particulières de la méthode des DNP-aminoacides.

a. DNP-aminoacides hydrosolubles d'un hydrolysat total de protéine

1. *Dinitrophénylation en milieu hydro-alcoolique* (voir paragraphe C, b, 2, p. 234)

Les DNP-aminoacides non éthérosolubles du milieu sont l'α-mono-DNP-arginine et la di-DNP-histidine. La di-DNP-histidine peut être extraite par l'acétate d'éthyle. On peut donc être amené soit à séparer l'α-mono-DNP-arginine et la di-DNP-histidine, soit à chromatographier l'α-mono-DNP-arginine seule. Ce problème peut être facilement résolu par une chromatographie unidimensionnelle dans le système "toluène" (voir Fig. 11). Malgré la simplicité apparente de la question, de nombreuses difficultés techniques peuvent se présenter au cours de sa résolution. La variabilité des R_F de ces composés, la présence d'artefacts colorés sont les principales causes d'erreur. Il est donc indispensable d'effectuer la chromatographie unidimensionnelle sur une feuille de papier Whatman No. 1 distincte et non pas, comme l'avait indiqué LEVY[43], sur le chromatogramme qui est utilisé pour la séparation des DNP-aminoacides

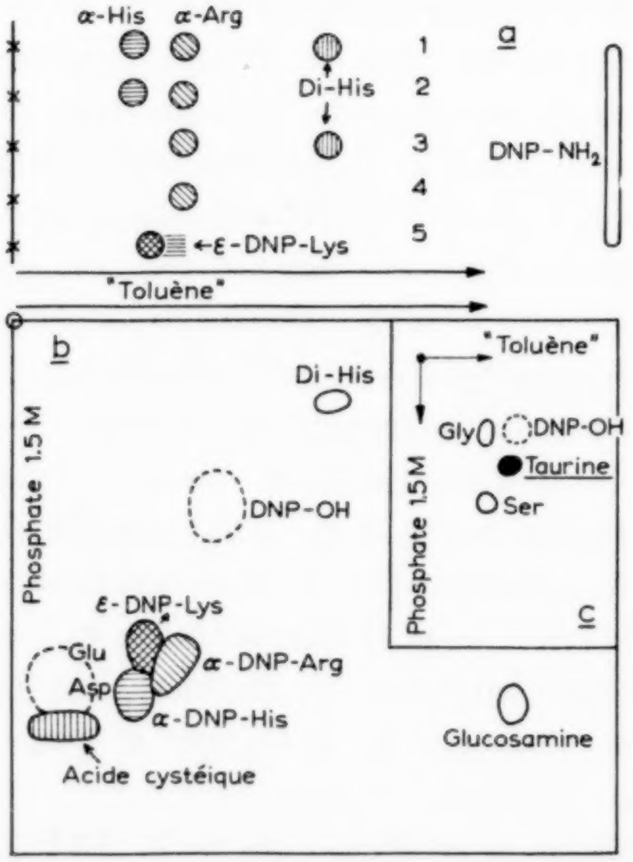


Fig. 11. Chromatographie sur papier des DNP-aminoacides hydrosolubles. (*a*) Chromatographie unidimensionnelle des DNP-aminoacides hydrosolubles (système solvant "toluène"). 1 = mélange des dérivés témoins; 2 = hydrolysat total après dinitrophénylation en milieu aqueux (technique de LEVY, paragraphe C, b, 1, p. 232); 3 = hydrolysat total après dinitrophénylation en milieu hydro-alcoolique (paragraphe C, b, 2, p. 234); 4 = hydrolysat total après extraction de la di-DNP-histidine par l'acétate d'éthyle (voir Tableau II); 5 = présence d'ε-DNP-lysine (et éventuellement d'autres dérivés dinitrophénylés hydrosolubles) après hydrolyse totale d'une DNP-protéine. (*b*) Carte chromatographique bidimensionnelle ("toluène" et "phosphate 1.5 *M*") de dérivés dinitrophénylés hydrosolubles. (*c*) Position de la DNP-taurine (hydrosoluble) sur la carte chromatographique "toluène" et "phosphate 1.5 *M*".

éthérosolubles (les deux dépôts de substances éthéro- et hydrosoluble se faisant aux deux extrémités de la feuille, la bandelette contenant les DNP-aminoacides hydrosolubles étant découpée ensuite avant la mise en route de la deuxième dimension dans le phosphate 1.5 *M*).

Sur cette feuille séparée, il est également indispensable de placer des témoins latéraux et internes d'α-mono-DNP-arginine et de di-DNP-histidine et d'effectuer sur le papier une révélation de Sakaguchi*, réaction qui donne une coloration rouge orangé avec l'α-mono-DNP-arginine.

2. *Dinitrophénylation en milieu aqueux* (technique de LEVY; voir paragraphe C, b, 1, p. 232)

Dans ces conditions, les DNP-aminoacides hydrosolubles peuvent être l'α-mono-DNP-arginine et l'α-mono-DNP-histidine, celle-ci n'étant pas extractible par l'acétate d'éthyle. La séparation peut s'effectuer également en chromatographie unidimensionnelle dans le système "toluène" sur une feuille distincte, en présence de témoins latéraux et internes. Enfin, il est indispensable de vérifier la nature des dérivés dinitrophénylés par la réaction de Sakaguchi pour l'α-mono-DNP-arginine et par la réaction de Pauly** pour l'α-mono-DNP-histidine.

b. DNP-aminoacides hydrosolubles d'un hydrolysat de DNP-protéine ou de DNP-peptide

(Problème de l'identification d'un résidu d'acide aminé en position terminale)

1. *Protéine*

Dans le cas d'une protéine, l'histidine et l'arginine en position N-terminale peuvent donner des dérivés hydrosolubles, la di-DNP-histidine (réaction de Pauly négative) et éventuellement l'α-mono-DNP-histidine (réaction de Pauly positive), et l'α-mono-DNP-arginine (réaction de Sakaguchi positive). Dans le cas d'une protéine oxydée, la cystine éventuellement en position terminale est oxydée en acide cystéique et la phase hydrosoluble de l'hydrolysat de la DNP-protéine oxydée peut également contenir de l'acide DNP-cystéique.

Mais l'hydrolysat contient obligatoirement d'autres dérivés dinitrophénylés: l'ε-DNP-lysine (composé jaune, donnant une réaction brune à la ninhydrine), l'imidazole-DNP-histidine (composé peu coloré, donnant une réaction brune à la ninhydrine et présentant une fluorescence sombre en lumière de Wood, ne donnant pas la réaction de Pauly), l'O-DNP-tyrosine (composé incolore, donnant une réaction violette à la ninhydrine, présentant une fluorescence sombre en lumière de Wood). Éventuellement, on peut trouver également de la S-DNP-cystéine.

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* *Technique de la réaction de Sakaguchi recommandée*: pulvériser le chromatogramme avec une solution de 8-hydroxyquinoléine à 0.1% dans l'acétone; après séchage, pulvériser une solution d'hypobromite de sodium préparée extemporanément en dissolvant 0.2 ml de brome dans 100 ml de soude 0.5 *N*; les taches de DNP-arginine virent au rouge rosé.

** *Technique de la réaction de Pauly recommandée*: pulvériser le chromatogramme avec le mélange à parties égales des deux réactifs suivants: *réactif a*: *p*-anisidine 1 g, acide chlorhydrique pur 1 ml, éthanol absolu 100 ml; *réactif b*: nitrite d'amyle 10 g, éthanol absolu q.s.p. 100 ml; attendre 3 à 5 min le séchage du papier à la température du laboratoire, puis pulvériser une solution de potasse à 1% dans l'éthanol; l'α-mono-DNP-histidine et l'histidine donnent des taches rouge brique sur un fond jaune orangé.

Le problème à résoudre peut donc être relativement complexe, d'autant plus que les quantités d'ε-mono-DNP-lysine sont toujours importantes par rapport aux quantités de dérivés dinitrophénylés pouvant provenir d'un résidu en position N-terminale.

La question peut être abordée de plusieurs façons. On peut essayer de séparer l'ensemble des composés par une technique chromatographique. Il est possible également de simplifier le mélange avant de le soumettre à la chromatographie.

(*i*) *Séparations chromatographiques des dérivés dinitrophénylés hydrosolubles.* Les systèmes solvants préconisés peuvent être soit ceux de la chromatographie classique des acides aminés, soit ceux de la chromatographie des dérivés dinitrophénylés éthérosolubles.

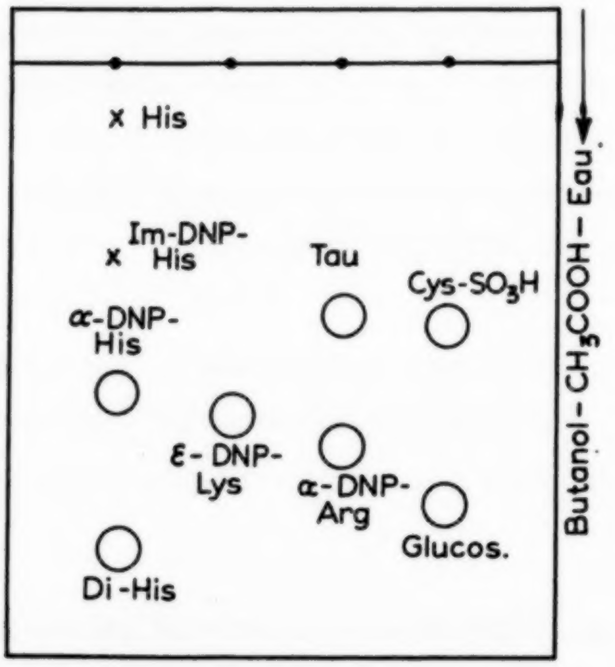

Fig. 12. Chromatographie sur papier des DNP-aminoacides hydrosolubles. Système solvant: butanol–acide acétique–eau (4:1:5). Les croix indiquent l'emplacement de l'histidine libre (His) et de l'imidazole-DNP-histidine (Im-DNP-His), composé très faiblement coloré en jaune. Cys-SO$_3$H = acide DNP-cystéique.

Les *systèmes butanoliques acides* peuvent être employés avec profit: système butanol–acide acétique–eau (4:1:5) de PARTRIDGE, préparé 24 heures avant son usage (SANGER ET TUPPY[80]) (voir Tableaux VI et VII et Fig. 12); système *n*-butanol–acide acétique–eau (3:1:1) (INGRAM ET SALTON[36]) en chromatographie ascendante*; système *n*-butanol–acide formique–eau (75:15:10) (MARGOLIASH[54], ACHER[1]) (voir Tableau VII). Dans le système butanol–acide acétique–eau (250:60:250 v/v) de WOIWOD[93], la δ-mono-DNP-ornithine migre moins vite que l'ε-mono-DNP-lysine

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* Ce système permet de séparer l'ε-mono-DNP-lysine et l'acide mono-DNP-diaminopimélique. Ces deux dérivés peuvent également être séparés en électrophorèse sur papier Whatman No. 3 MM en tampon de pH 6.4 (pyridine–acide acétique–eau: 10:0.4:90) sous 20 V/cm pendant 1 h (INGRAM ET SALTON[36]).

(NEWTON ET ABRAHAM[62]). La variabilité des R_F suivant les conditions expérimentales oblige à l'utilisation de témoins internes latéraux et de colorations spécifiques (réactions de Pauly et de Sakaguchi).

TABLEAU VI

R_F DES DÉRIVÉS DINITROPHÉNYLÉS HYDROSOLUBLES

Papier Whatman No. 4; solvant butanol–acide acétique–eau (4:1:5) préparé 24 h avant usage.

	R_F	*Couleur des taches*	*Réaction à la ninhydrine*	*U.V.*
O-DNP-tyrosine	0.84	incolore	violet	sombre
α-DNP-arginine	0.81	jaune	pas de réaction	
ε-DNP-lysine	0.77	jaune	brun	
Leucine*	0.67	—	—	
Imidazole-DNP-histidine	0.57	(?)	brun	sombre
Valine*	0.49	—	—	
Acide DNP-cystéique	0.42	jaune	—	

* Les R_F de ces acides aminés sont donnés à titre de comparaison.

TABLEAU VII

R_F DES DÉRIVÉS DINITROPHÉNYLÉS HYDROSOLUBLES DANS QUELQUES SYSTÈMES SOLVANTS

	Butanol–acide formique		*Butanol–acide acétique*		*Phénol (NH_3)*
	MARGOLIASH[54]	ACHER[1]	SANGER ET TUPPY[80]	*Résultats personnels*	
Histidine	0.02			0.09	
α-DNP-histidine	0.09			0.57	
Imidazole-DNP-histidine	0.16	0.24	0.57	0.33	
Di-DNP-histidine	0.63	0.75		0.83	0.87
ε-DNP-lysine		0.56	0.77	0.60	0.90
α-DNP-arginine		0.65	0.81	0.65	0.93
Acide DNP-cystéique*			0.42	0.45	0.24
Taurine				0.43	

* L'acide DNP-cystéique n'est pas adsorbé sur les colonnes de talc comme les autres dérivés dinitrophénylés hydrosolubles (voir paragraphe D, d, 1, p. 242).

Les *systèmes phénoliques* peuvent également être essayés, notamment le phénol saturé d'eau en atmosphère d'ammoniaque à 3% (v/v) et d'acide cyanhydrique (voir Tableau VII), qui permet une séparation satisfaisante de l'acide DNP-cystéique de l'ensemble des autres dérivés dinitrophénylés hydrosolubles. En chromatographie bidimensionnelle [butanol–acide acétique–eau (4:1:5) en première dimension et phénol (NH_3 3%) ou *m*-crésol–phénol–tampon borate de pH 9.3 (25:25:7)* en seconde dimension] l'acide DNP-cystéique est nettement séparé.

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* Tampon borate: 200 ml d'acide borique 0.1 *N* et 113.5 ml de NaOH 0.1 *N*. Le tampon est pulvérisé également sur le papier avant la mise en route de la seconde dimension, sauf dans la zone du chromatogramme où se trouvent les acides aminés séparés par la première dimension (LEVY ET CHUNG[44]). Dans la carte chromatographique des acides aminés et des DNP-aminoacides hydrosolubles donnée par FRAENKEL-CONRAT, HARRIS ET LEVY[28], la position de la di-DNP-histidine est inexacte (voir Fig. 12).

Les systèmes solvants décrits pour les dérivés dinitrophénylés éthérosolubles peuvent être aussi employés, notamment le système alcool *tert.*-amylique–tampon phtalate de pH 6 de Blackburn et Lowther (voir Fig. 13) ou le système "toluène" de Biserte et Osteux (voir Fig. 11).

La chromatographie bidimensionnelle "toluène–phosphate 1.5 *M*" a également été préconisée. Mais le résultat n'est pas très satisfaisant; la séparation de l'arginine et de l'ε-mono-DNP-lysine n'est pas très poussée* et ce couplage ne donne pas de meilleurs résultats que la chromatographie unidimensionnelle.

L'électrophorèse sur papier peut aussi résoudre facilement quelques problèmes. Par exemple, la séparation de l'α-DNP-arginine et de l'ε-DNP-lysine peut être réalisée en électrophorèse sur papier en milieu ammoniaque *N* dans un appareil "en toit" (type Durrum)(voir Fig. 14). De même, la séparation de l'acide DNP-cystéique et de l'ε-mono-DNP-lysine, extraits de l'hydrolysat par le *n*-butanol, peut être obtenue en électrophorèse sur papier Whatman No. 3 imbibé par un tampon phosphate 0.1 *M* à pH 7.0 (Anfinsen, Sela et Tritch[3]).

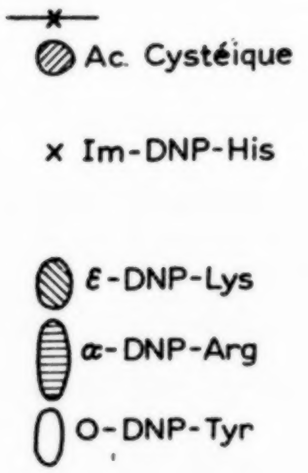
Ac. Cystéique
× Im-DNP-His
ε-DNP-Lys
α-DNP-Arg
O-DNP-Tyr

Fig. 13. Chromatographie des DNP-aminoacides hydrosolubles. Système solvant alcool-*tert.*-amylique–phtalate de Blackburn et Lowther (d'après Fraenkel-Conrat, Harris et Levy[28]).

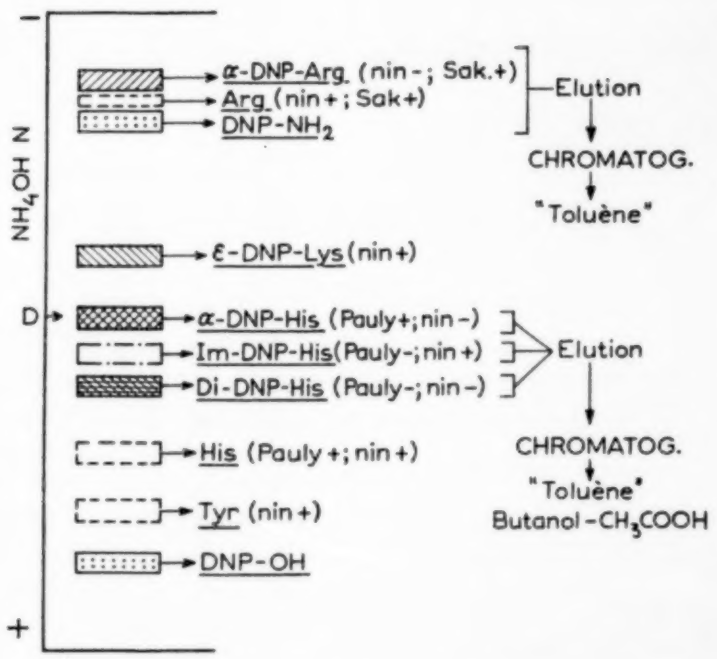
−
α-DNP-Arg (nin -; Sak.+)
Arg (nin +; Sak+)
DNP-NH$_2$
Elution
CHROMATOG.
"Toluène"
NH$_4$OH N
ε-DNP-Lys (nin+)
D
α-DNP-His (Pauly+; nin -)
Im-DNP-His (Pauly-; nin +)
Di-DNP-His (Pauly-; nin -)
Elution
His (Pauly +; nin +)
CHROMATOG.
"Toluène"
Butanol-CH$_3$COOH
Tyr (nin +)
DNP-OH
+

Fig. 14. Séparation électrophorétique des DNP-aminoacides hydrosolubles. Papier Whatman No. 1 ou No. 3; tampon NH$_4$OH *N*; appareil d'électrophorèse "en toit" type Durrum. D = point de départ de l'électrophorèse; nin = réaction à la ninhydrine; Sak = réaction de Sakaguchi; Pauly = réaction de Pauly; DNP-OH = dinitrophénol; DNP-NH$_2$ = dinitraniline. Les taches entourées par des traits pointillés ne sont pas jaunes à l'examen direct. Après élution des diverses taches séparées en électrophorèse, les éluats sont étudiés en chromatographie unidimensionnelle.

(*ii*) *Simplification du mélange des dérivés dinitrophénylés hydrosolubles avant l'étude chromatographique.*

(α) *Cas de l'α-DNP-arginine.* On peut éliminer les acides aminés de l'hydrolysat (tous les acides aminés constitutifs, plus quelques molécules de lysine ou d'histidine

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* La carte chromatographique publiée par Davies et Harris[22] est incomplète et partiellement inexacte.

Bibliographie p. 269/271.

non réactives et non dinitrophénylées) sur une colonne de talc (SANGER, voir paragraphe D, d, 1, p. 241). L'éluat de la colonne de talc, qui contient les dérivés dinitrophénylés hydrosolubles, est traité par le fluorodinitrobenzène (ROVERY, FABRE ET DESNUELLE[75]). Dans ces conditions, l'ε-mono-DNP-lysine devient de la di-DNP-lysine, l'O-DNP-tyrosine de la di-DNP-tyrosine, l'imidazole-DNP-histidine de la di-DNP-histidine, tandis que le dérivé dinitrophénylé de l'arginine (α-mono-DNP-arginine) n'est pas modifié. Après cette seconde dinitrophénylation, l'extraction éthérée du résidu enlève la di-DNP-lysine et la di-DNP-tyrosine, tandis que la phase aqueuse contient de la di-DNP-histidine et l'α-mono-DNP-arginine provenant d'un acide aminé terminal de la chaîne peptidique. La phase aqueuse est chromatographiée dans le système butanol–acide acétique–eau (4:1:5) (voir plus haut) et l'identification certaine de l'α-mono-DNP-arginine est obtenue au moyen de la réaction de Sakaguchi.

Un autre mode opératoire a également été proposé par BAILEY[4]. L'ε-mono-DNP-lysine est transformée ici en ε-mono-DNP-α-méthoxycarbonyllysine (ε-DNP-α-MC-lysine) qui est éthérosoluble. Le mode opératoire est le suivant. Après extraction des DNP-aminoacides éthérosolubles, la phase aqueuse (équivalente à 0.2 g de protéine) est évaporée à siccité, dissoute dans l'acide chlorhydrique 0.1 *N* et passée sur une colonne de talc (voir BAILEY ET BETTELHEIM[5] et paragraphe D, d, 1, p. 242). Les aminoacides hydrosolubles sont élués à l'éthanol chlorhydrique (éthanol: 4 vol.; HCl *N*: 1 vol.). Ils sont dissous dans 2–3 ml d'un mélange bicarbonate–carbonate de pH 8.9 (mélange de 20 ml de bicarbonate de sodium à 10% et de 5 ml de carbonate de sodium à 10%). A la solution portée à 20°, on ajoute à quatre reprises 0.02 ml de chlorure de méthoxycarbonyl à des intervalles de 10 min et sous une agitation vigoureuse. Le mélange est acidifié avec de l'acide chlorhydrique, extrait 4 fois à l'éther pour éliminer l'ε-DNP-α-MC-lysine. La phase aqueuse est chromatographiée comme précédemment.

(*β*) *Cas des dérivés de l'histidine.* Pour résoudre ce problème, il est impossible d'utiliser la technique décrite dans le cas de l'α-DNP-arginine, parce que, au cours de la seconde dinitrophénylation, tous les dérivés de l'histidine, en position intrapeptidique ou terminale, peuvent être transformés en di-DNP-histidine.

Pour aborder cette question très délicate, on peut avoir recours aux extractions sélectives, comme l'extraction éthérée continue du résidu aqueux suivant les modalités décrites et avec l'appareillage préconisé par MILLS[58], ou à l'extraction à l'acétate d'éthyle.

L'utilisation de la chromatographie unidimensionnelle en présence de témoins internes et latéraux, avec mise en œuvre de colorations spécifiques (réactions de Pauly, ninhydrine), constitute l'étape ultime de l'identification, qui n'est pas toujours couronnée de succès.

En conclusion, l'identification des dérivés dinitrophénylés hydrosolubles dérivant d'acides aminés en position N-terminale est toujours délicate et, avant de conclure à leur existence, il est indispensable de s'entourer de garanties nombreuses et indiscutables. Il faut employer plusieurs systèmes solvants et réaliser une électrophorèse sur papier (Fig. 14).

(*iii*) *Artefacts de la fraction hydrosoluble.* Un certain nombre d'artefacts peuvent être trouvés dans la fraction hydrosoluble.

Bailey[4], notamment, a signalé la présence d'une impureté de couleur brune qui peut être facilement séparée de l'α-mono-DNP-arginine (si celle-ci est présente) par passage sur une petite colonne de silice acide éluée par de la méthyléthylcétone (Sanger[76]).

Nous avons également trouvé assez souvent sur les chromatogrammes des taches colorées en jaune, de nature inconnue. C'est pour cette raison qu'il est absolument indispensable d'utiliser des témoins latéraux et internes et des réactions de coloration spécifiques pour localiser les DNP-aminoacides hydrosolubles.

Signalons également un artefact qui peut gêner l'identification de l'acide DNP-cystéique*. En chromatographie bidimensionnelle butanol–acide acétique et phénol aqueux, les R_F de ces deux composés sont les suivants, d'après Thompson[88]:

	R_F *artefact*	R_F *acide DNP-cystéique*
Butanol–acide acétique	0.29	0.18
Phénol	0.58	0.38

2. *Problèmes particuliers de séparation*

(*i*) *Séparation des dérivés monodinitrophénylés des acides diaminés.* Dans la chimie des polypeptides bactériens, on peut trouver non seulement de la lysine, mais aussi de l'ornithine et de l'acide α,γ-diaminobutyrique. La séparation des dérivés dinitrophénylés de ces acides diaminés est très délicate: nous avons signalé plus haut l'impossibilité de séparer les dérivés dinitrophénylés éthérosolubles de la lysine, de l'ornithine et de l'acide α,γ-diaminobutyrique. La séparation des dérivés monodinitrophénylés soit en position α, soit en position ε, est également très difficile. Le procédé proposé par Newton et Abraham[62] pour la séparation de l'ε-mono-DNP-lysine et de la δ-mono-DNP-ornithine dans le butanol–acide acétique–eau (250:60:250) est peu efficace, même en présence de témoins latéraux.

Des résultats plus satisfaisants peuvent être obtenus en électrophorèse sur papier.

(α) *Séparation de l'ε-mono-DNP-lysine, de la δ-DNP-ornithine et de l'acide γ-mono-DNP-diaminobutyrique.* Électrophorèse en tampon borate 0.02 M de pH 9.1 (Lockhart et Abraham[50]) sur papier Arches 304 ou Whatman No. 3, dans un appareil "en toit" (type Durrum); 10 V/cm pendant 17 heures (voir Fig. 15a).

(β) *Séparation de l'α-mono-DNP-lysine, de l'α-mono-DNP-ornithine et de l'acide α-mono-DNP-diaminobutyrique.* Electrophorèse en milieu ammoniaque N sur papier Whatman No. 3 dans un appareil "en toit" (type Durrum); 8–9 V/cm pendant 16 heures (voir Fig. 15b). Cette électrophorèse ne sépare pas l'acide α-mono-DNP-diaminobutyrique de l'acide γ-mono-DNP-diaminobutyrique, ni l'α-mono-DNP-ornithine de la δ-mono-DNP-ornithine. L'ε-mono-DNP-lysine se confond avec l'α-mono-DNP-ornithine.

Le couplage de 2 électrophorèses unidimensionnelles, l'une en milieu NH_4OH N et l'autre ensuite, après élution, en tampon borate 0.02 M de pH 9.1, permet de résoudre tous ces problèmes.

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* Ce problème s'est posé récemment au sujet des acides aminés terminaux de la sérumalbumine. Thompson a démontré que la substance identifiée par Titani *et al.*[89] était un artefact.

(γ) *Séparation des dérivés monodinitrophénylés en position* α *ou* ω. Les systèmes solvants chromatographiques ne permettent pas la résolution de ces différents composés. La séparation des dérivés *d'une même série*: α-mono-DNP-lysine et ε-mono-DNP-lysine; α-mono-DNP-ornithine et δ-mono-DNP-ornithine; acide α-mono-DNP-diaminobutyrique et acide γ-mono-DNP-diaminobutyrique, est facilement obtenue en électrophorèse sur papier dans le tampon borate 0.02 *M* (pH 9.1) (voir Fig. 15a).

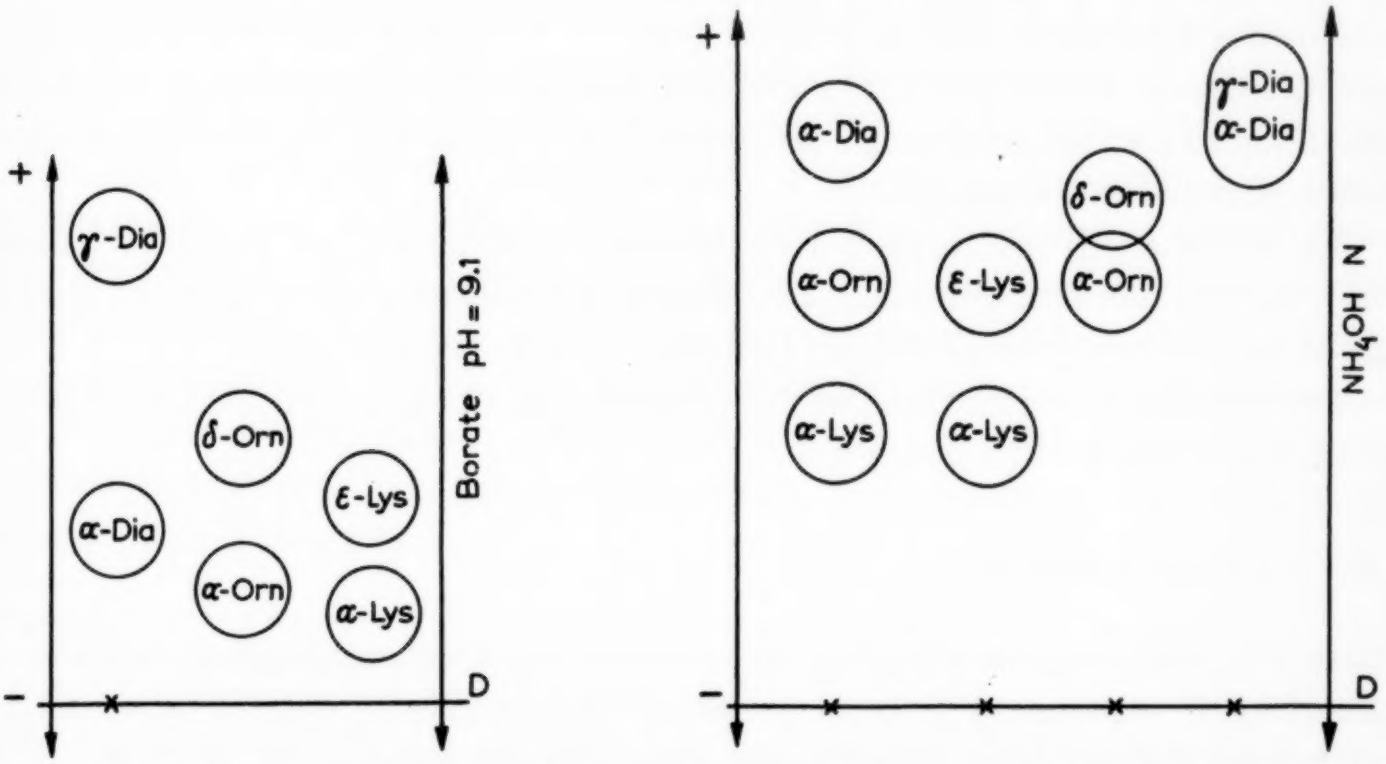

Fig. 15. (*a*) Électrophorèse sur papier des dérivés dinitrophénylés d'acides diaminés. Électrophorèse "en toit" type Durrum; tampon borate 0.02 *M* de pH 9.1; papier Arches 304 ou Whatman No. 3; 10 V/cm; 17 h. Distance parcourue par l'acide α-DNP-diaminobutyrique = 6 cm; par l'acide γ-DNP-diaminobutyrique = 16 cm. (*b*) Électrophorèse sur papier des dérivés dinitrophénylés des acides diaminés. Électrophorèse "en toit" type Durrum; papier Whatman No. 3; tampon NH_4OH *N*; 8–9 V/cm; 16 h. Distance parcourue par l'α-DNP-lysine = 5 cm.

(*ii*) *Séparation de la DNP-glucosamine et de la DNP-chondrosamine.* Ces deux dérivés peuvent être séparés facilement dans le système solvant butanol–éthanol–eau (4:1:5) sur papier Whatman No. 1. Les R_F de ces substances sont respectivement: DNP-glucosamine 0.75; DNP-chondrosamine 0.61 (KENT *et al.*[38]).

Sur le chromatogramme bidimensionnel "toluène–phosphate 1.5 *M*", la DNP-glucosamine est très nettement séparée de tous les autres dérivés (voir Fig. 11).

(*iii*) *DNP-taurine.* Elle peut être également chromatographiée dans le système butanol–acide acétique et dans le système "toluène" (voir Fig. 11).

I. CHROMATOGRAPHIE DES DNP-PEPTIDES

La chromatographie des DNP-peptides pose des problèmes très particuliers et il est difficile de présenter un plan précis de séparation valable dans tous les cas*.

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* THOMPSON[86] et MCFADDEN ET SMITH[53] ont proposé des plans de fractionnement sur des colonnes de Celite tamponnée. La distribution à contre-courant peut également donner des résultats très satisfaisants (BATTERSBY ET CRAIG[6], REDFIELD ET ANFINSEN[70]).

Les DNP-peptides proviennent le plus souvent d'un hydrolysat partiel acide ou enzymatique d'une DNP-protéine. L'hydrolysat partiel peut être extrait successivement à l'éther sulfurique (3 fois), à l'acétate d'éthyle (3 fois), au *n*-butanol (1 fois). La phase aqueuse résiduelle peut contenir encore des peptides dinitrophénylés (Li *et al.*[49]).

Le comportement des DNP-oligopeptides se rapproche dans une certaine mesure de celui des DNP-aminoacides. D'autre part, la séparation des DNP-peptides est assez rapidement limitée par leur insolubilité dans les solvants organiques ou dans les milieux aqueux acides ou alcalins. On pourra donc essayer tous les systèmes solvants décrits pour les acides aminés.

Le système solvant suivant a également été employé, sur papier Whatman No. 3: *sec.*-butanol–*tert.*-butanol–2,4,6-collidine–ammoniaque–acide acétique glacial–eau (60:20:20:2:0.25:100 vol./vol.) (Redfield et Anfinsen[70]).

Des couplages intéressants ont été proposés par Cole, Li, Harris et Pon[19] et par Li, Cole, Chung et Leonis[49].

1. *DNP-peptides éthérosolubles*

Séparations unidimensionnelles dans les systèmes alcool *tert.*-amylique–alcool isoamylique–NH_4OH aqueuse 3% (1:1:2); alcool *tert.*-amylique–NH_4OH aqueuse 3% (1:1); alcool *tert.*-amylique–alcool isoamylique–NH_4OH aqueuse 3% (2:1:3).

2. *DNP-peptides solubles dans l'acétate d'éthyle*

Chromatographie bidimensionnelle avec l'alcool *tert.*-amylique–NH_4OH aqueuse 3% (1:1) en première dimension et un tampon phosphate 0.8 *M* de pH 6.7 en seconde dimension; ou chromatographie unidimensionnelle dans le système alcool *tert.*-amylique–alcool isoamylique–ammoniaque 3% (2:1:3).

3. *DNP-peptides solubles dans le n-butanol et dans la phase aqueuse*

Chromatographies unidimensionnelles dans le système alcool *tert.*-amylique–alcool isoamylique–ammoniaque 3% (2:1:3) et phosphate 1.6 *M* de pH 7.

Il faut également signaler les possibilités séparatives offertes par l'électrophorèse sur papier, notamment en tampon saturé d'urée, qui diminue l'adsorption des DNP-peptides sur le papier (solution 8 *M* en urée et tampon acétate–collidine 0.04 *M* de pH 7.9) (Redfield et Anfinsen[70]). Les électrophorèses à haut potentiel sont conduites sous 800 V sur papier Whatman No. 3 sous toluène (Michl[56]).

J. CHROMATOGRAPHIE QUANTITATIVE DES DNP-AMINOACIDES

La chromatographie des DNP-aminoacides peut être facilement rendue quantitative. Après élution de la tache, l'éluat est dosé directement par spectrophotométrie.

a. Dosage des acides aminés d'un hydrolysat
(méthode de LEVY[43])

1. *Élution des taches*

Pour l'hydrolyse et la formation des DNP-aminoacides, voir paragraphes C, a et C, b, pp. 232 *et seq.*). Après la chromatographie des DNP-aminoacides éthérosolubles (chromatogramme bidimensionnel réalisé à partir des DNP-aminoacides correspondant à 0.1–0.2 mg de protéine) et hydrosolubles (chromatogramme unidimensionnel réalisé à partir des DNP-aminoacides correspondant à 0.2–0.4 mg de protéine) (voir pp. 246, 256), les taches sont soigneusement découpées et les surfaces de papier correspondantes sont placées dans des petits tubes à centrifuger avec 4 ml de bicarbonate de sodium à 1%. Les tubes sont chauffés à 50–55° dans un bain-marie pendant 20 min pour parfaire l'élution, puis secoués et centrifugés. Trois surfaces de papier servant de "blancs" (3 × 4 cm) sont également découpées dans le chromatogramme, dans des régions voisines de la DNP-leucine et des acides DNP-dicarboxyliques et au-dessus du dinitrophénol. Après refroidissement, l'éluat est versé dans une cuve de quartz de 1 cm d'épaisseur.

2. *Dosage*

La densité optique est lue au spectrophotomètre à 360 mμ (sauf pour la proline et l'hydroxyproline où la lecture se fait à 385 mμ, pour les DNP-peptides à 350 mμ et pour l'ε-mono-DNP-lysine à 390 mμ), contre un blanc contenant de l'eau.

Les densités optiques des tubes contenant les "blancs papier" sont également mesurées: les résultats sont de l'ordre de 0.001–0.002 par cm^2. Les corrections appropriées sont faites pour chaque tache d'après sa surface. La densité optique de la tache "acide aspartique + acide glutamique" est mesurée dans sa totalité.

A partir d'un second chromatogramme effectué avec le couplage "toluène–phosphate 2.5 *M*", on peut déterminer les proportions d'acide glutamique et d'acide aspartique.

3. *Expression des résultats*

Les récupérations en quantités absolues varient d'un chromatogramme à l'autre. Les résultats sont donc exprimés sur une base commune en fractions molaires.

Afin de convertir les densités optiques en rapports molaires, on les multiplie par les facteurs de LEVY (voir Tableau VIII)* qui, étant donnée la précision de la méthode (± 4%), sont indépendants de la composition du mélange analysé.

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* Les facteurs de LEVY ont été déterminés par cet auteur pour les hydrolysats d'insuline. Ils ne sont pas obligatoirement entièrement valables pour les autres protéines. BROMER *et al.*[17], par exemple, ont calculé de nouveaux facteurs pour le glucagon. En première approximation, ils appliquent à l'hydrolysat protéique les facteurs de LEVY. A partir des données quantitatives ainsi obtenues, ils préparent un mélange d'acides aminés qui ressemble au glucagon, ils le soumettent aux opérations d'hydrolyse et le traitent comme un hydrolysat protéique. De cette façon, de nouveaux facteurs de destruction peuvent être calculés. Cette opération est répétée plusieurs fois pour obtenir une plus grande précision des facteurs de correction. Dans l'ensemble, les facteurs de BROMER sont plus élevés (17% environ) que ceux de LEVY. Notamment, l'histidine et la méthionine subissent des pertes plus grandes que celles signalées par LEVY.

TABLEAU VIII

FACTEURS DE CORRECTION UTILISÉS AU COURS DU DOSAGE DES DNP-AMINOACIDES

	Facteurs de Levy[13]	*Facteurs de* Bromer[17]
Glycocolle	1.03	1.33
Sérine	0.97	1.32
Thréonine	1.02	1.22
Proline	0.93	—
Alanine	1.09	1.28
Méthionine	1.21	2.12
Valine	0.99	1.20
Leucine	1.10	—
Phénylalanine	1.03	1.20
Lysine	0.64	0.70
Arginine	1.06	1.38
Histidine	1.62	2.19
Acide aspartique	0.99	1.10
Acide glutamique	0.94	1.20
Cystine	0.56	—
Tyrosine	1.54	1.60

Les chromatogrammes sont effectués en triple. Plusieurs hydrolysats de durées différentes peuvent être analysés (24 et 48 heures d'hydrolyse, par exemple).

Toutes les lectures de densité optique, multipliées par les facteurs de Levy, sont additionnées. Pour chaque somme ainsi obtenue, on peut calculer un facteur pour lequel cette somme est égale à l'unité.

En éliminant les valeurs aberrantes qui proviennent indubitablement d'erreurs techniques ou d'imperfections chromatographiques, on peut calculer une moyenne de tous les résultats et les exprimer en fractions molaires (voir Tableau IX). En général, les résultats qui sont les plus variables sont ceux de la tyrosine (variabilité dans la synthèse), de la méthionine (dégradation possible) et de l'histidine (variabilité dans la synthèse et extraction délicate). D'après Levy, les rapports molaires sont reproductibles à 2–3% près.

A partir de ces valeurs, on peut calculer le nombre de résidus d'acides aminés par poids moléculaire minimum de la protéine, en divisant la moyenne des valeurs des fractions molaires par celle d'entre elles qui conduit au résultat le plus voisin d'un nombre entier pour le plus grand nombre possible d'acides aminés (voir Tableau IX, exemple de la leucine). Les valeurs de la sérine sont corrigées (10%) par suite de la destruction partielle de cet aminoacide après 24 heures d'hydrolyse (Rees[71]). Ces valeurs sont ensuite arrondies à la valeur unitaire la plus approchée (voir Tableau IX).

Il est évident qu'en partant des résultats en fractions molaires, on peut également connaître le nombre de résidus d'acides aminés pour 100 acides aminés de la molécule. Pour obtenir ce résultat, on additionne les lectures de densité optique multipliées par les facteurs de Levy. Pour chaque somme, on peut trouver un facteur de correction tel qu'elle devienne égale à 100 (au lieu de 1 dans le calcul précédent). En multipliant tous les nombres par ce facteur, on peut donc exprimer les résultats sur la base de 100 résidus d'acides aminés.

TABLEAU IX

COMPOSITION EN ACIDES AMINÉS D'UNE PRÉPARATION D'α-CORTICOTROPHINE

(D'après LEVY *et al.*[46])

Acide aminé	*Résultats en fractions molaires*			*Moyenne des fractions molaires*	*Déviation de la moyenne (%)*	*Nombre de résidus d'acides aminés par poids mol. minimum*	*Nombre de résidus (valeur arrondies)*
	I	*II*	*III*				
Ac. aspartique	0.0392	0.0503	0.0491	0.0462	10.1	1.72	2
Ac. glutamique	0.1345	0.1273	0.1282	0.1300	2.3	4.85	5
Sérine	0.0689	0.0690	0.0702	0.0694	0.9	2.85	3
Glycocolle	0.0837	0.0818	0.0842	0.0832	1.2	3.10	3
Alanine	0.0747	0.0753	0.0753	0.0751	0.3	2.80	3
Proline	0.1107	0.1084	0.1087	0.1093	0.9	4.08	4
Valine	0.0798	0.0798	0.0816	0.0804	1.0	2.99	3
Méthionine	0.0235	0.0211	0.0212	0.0219	4.7	0.82	1
Leucine	0.0291	0.0246	0.0268	0.0268	5.7	1.00	1
Phénylalanine	0.0822	0.0809	0.0823	0.0818	0.8	3.05	3
Tyrosine	0.0608	0.0647	0.0607	0.0621	2.8	2.32	2
Lysine	0.1049	0.1073	0.1018	0.1047	1.8	3.91	4
Histidine	0.0268	0.0272	0.0273	0.0271	2.1	1.01	1
Arginine	0.0810	0.0821	0.0825	0.0819	0.4	3.06	3
Tryptophanne*							1
NH_3 amidé							2
Total	0.9998	0.9998	0.9999	0.9999	2.5	37.6	41

* Dosé par la méthode de GOODWIN ET MORTON[30].

En tenant compte du poids moléculaire de la molécule, on peut calculer le nombre de résidus de chaque acide aminé présent dans la molécule entière. Connaissant le nombre de chaque résidu pour 100 acides aminés, on multiplie ce nombre par le poids moléculaire de l'acide aminé correspondant. On fait la somme de tous les résultats. De la somme ainsi obtenue, on enlève la valeur de 1782, correspondant à 99 molécules d'eau. On calcule le rapport entre le poids moléculaire réel et le poids moléculaire partiel ainsi calculé. Le nombre de résidus pour 100 acides aminés multiplié par ce rapport donne le nombre de résidus pour l'ensemble de la molécule. Il suffit alors d'"arrondir" les valeurs pour connaître le nombre de résidus d'acide aminés par molécules (voir Tableau X). On peut également effectuer un calcul identique en tenant compte du nombre d'acides aminés trouvé pour le poids moléculaire minimum.

Enfin, s'il est nécessaire d'estimer les quantités absolues d'acides aminés présents dans le mélange, deux méthodes peuvent être appliquées (LEVY[43]): l'une, la moins précise (2–5%) consiste en une conversion directe de la densité optique lue en micromolécules au moyen d'un ensemble de coefficients d'extinction millimolaire. Ils ont la valeur 15.6/F ou F représente les facteurs du Tableau VIII. L'imprécision de ce procédé provient principalement des différences dans la récupération de la coloration à partir de plusieurs chromatogrammes. Ces différences n'ont pas d'importance dans la détermination des rapports molaires, car ces variations affectent tous les aminoacides d'une manière comparable.

La seconde méthode consiste à combiner la détermination plus précise (2–3%) des rapports molaires avec la détermination de la quantité totale d'acides aminés présents dans le mélange. Celle-ci peut être déduite de la consommation de soude au cours de la réaction de condensation (voir LEVY[43], LEVY *et al.*[46]).

TABLEAU X

COMPOSITION DE L'HÉMÉRYTHRINE DE *Sipunculus nudus*

(Résultats personnels)

	Nombre de résidus pour 100 acides aminés	*Nombre de résidus calculés d'après les valeurs de la colonne 1 sur la base d'un poids moléculaire de 66,000*	*Nombre de résidus dans l'hémérythrine (valeurs arrondies)*
Gly	4.42	25.37	25
Ser	4.22	24.33	24
Thr	4.33	24.85	25
Pro	5.62	32.25	32
Ala	7.40	42.47	42
Met	0.38	2.18	2
Val	6.80	39.03	39
Leu	14.10	80.93	81
Phe	11.62	66.69	67
Lys	8.15	46.78	47
Tyr	3.80	21.80	22
Arg	2.42	13.89	14
His	4.36	25.02	25
Asp	13.82	79.32	79
Glu	8.10	46.49	46
Cys	0.34	1.94	2
			572 résidus

TABLEAU XI

DESTRUCTION APPROXIMATIVE DES DÉRIVÉS DINITROPHÉNYLÉS AU COURS DE L'HYDROLYSE

(D'après PORTER[66])

	Temps (h)	*Quantité restante (%)*
DNP-alanine	12	80
DNP-arginine	12	90
Acide DNP-aspartique	24	60
Di-DNP-cystine	12	25
Acide DNP-glutamique	12	75
DNP-glycocolle	8	40
DNP-hydroxyproline	4	40
DNP-leucine	12	80
DNP-isoleucine	12	80
Di-DNP-lysine	8	95
ε-DNP-lysine	12	95
DNP-méthionine	12	75
DNP-phénylalanine	12	70
DNP-proline	2	10
DNP-sérine	12	90
DNP-thréonine	24	90
DNP-tryptophanne	12	90
DNP-tyrosine	12	75
DNP-valine	12	80

b. Dosage d'un DNP-aminoacide en position N-terminale

Une certaine quantité de DNP-aminoacide terminal est détruite au cours de l'hydrolyse (à titre d'indication, voir le Tableau XI) et, d'autre part, les récupérations chromatographiques entraînent également des pertes (par exemple, récupération chromatographique de l'acide DNP-aspartique et de l'acide DNP-glutamique: 90%; de la di-DNP-lysine: 80%; de l'acide DNP-cystéique: 90%) (REDFIELD ET ANFINSEN[70]).

Il faut donc tenir compte de ces pertes au cours des dosages. Pour les calculer, il est recommandé de soumettre à l'hydrolyse dans les mêmes conditions, en même temps que la protéine ou la DNP-protéine étudiée, une quantité déterminée du DNP-aminoacide identifié. La détermination de ces coefficients de destruction doit être effectuée plusieurs fois. A titre d'exemple, les pourcentages de récupération de la DNP-sérine hydrolysée en présence de l'α-corticotrophine et de son dérivé dinitrophénylé sont reproduits dans le Tableau XII. Il est remarquable de constater que la destruction de la DNP-sérine augmentée en présence de la corticotrophine est diminuée en présence de la DNP-corticotrophine (voir également les expériences de DESNUELLE *et al.*[24] sur l'acide DNP-aspartique de la sérumalbumine).

TABLEAU XII

RÉCUPÉRATION (%) DE LA DNP-SÉRINE APRÈS HYDROLYSE EN PRÉSENCE DE L'α-CORTICOTROPHINE ET DE SON DÉRIVÉ DINITROPHÉNYLÉ
(D'après LEVY ET LI[47])

Expérience No.	*Quantité de produit soumise à l'hydrolyse*			*Récupération de DNP-sérine* (%)*
	DNP-sérine (µmoles)	*α-Corticotrophine (µmoles)*	*DNP-α-corticotrophine (µmoles)*	
1	1.0	0	0	86
1	1.0	0	1.0	93
2	0.2	0	0	69–67
2	0.2	0.2	0	52–47
2	0.2	0	0.2	70–78

* Récupération de la DNP-sérine non hydrolysée = 93%.
Exp. 1 = hydrolyse pendant 7 h à 105° par HCl 5.7 *N* (tube scellé sous vide).
Exp. 2 = hydrolyse pendant 16 h à 110° par HCl 5.7 *N*.

L'interprétation des résultats du dosage d'un acide aminé en position terminale est très souvent délicate: il peut arriver que, malgré les corrections précédentes, la quantité récupérée ne représente qu'une assez faible partie de la molécule initiale.

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GAS CHROMATOGRAPHY

APPLICATION TO THE STUDY OF RAPID DEGRADATIVE REACTIONS IN SOLIDS

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Application of gas chromatography to the study of organic reaction kinetics is clearly a very promising and natural outcome of the recent development of this highly diversified analytical tool[1,2]. Reaction products of complex gas and liquid phase reactions can be rapidly and accurately determined regardless of the complexity of their composition. With suitable modification for the transferral of materials, any one of the commercially available chromatography units, combined with an infrared spectrometer or a mass spectrograph, provides a means for the complete analysis of practically any imaginable liquid or gas mixture. An application which has received relatively little attention, but which lends itself perfectly to chromatographic methods, involves the determination of the products of rapid degradative reactions of solids or of liquids with low vapor pressures. The essential feature of the method is one of carrying out the reaction directly in the stream of the chromatography carrier gas, through the influence of some external stimulus such as radiation (thermal, nuclear, or radio frequency), electric spark, or simply resistive heating. Examples of reactions which can be studied in this way include the ignition of explosives, the burning of solid propellants, the degradative processes induced in solids by ionizing radiations, and the rapid pyrolysis of organic compounds. In effect the method to be described can be used to study the reactions of any substance whose vapor pressure is small and which decomposes at least partially to gaseous products under the influence of applied stimuli rapidly enough that in an interval of time of a minute or less the reaction has run its course to such an extent that the nature of the reaction can be determined.

The method as it is used in the author's laboratory amounts to exposing an organic solid suspended in the helium carrier gas stream to very intense radiant energy similar in spectral distribution and radiant power to that which is emitted during a nuclear weapon detonation. Such radiation is produced in the laboratory by high-current carbon arcs[3]. High temperatures are developed in a solid in a very short time when it is exposed to this radiation, and depending upon the intensity of the radiation, the solid may be largely decomposed to vapors in a fraction of a second. Under the proper conditions, in air, the solid would ignite spontaneously and burn. While the subject of radiation ignition is quite beyond the scope of this paper, it should be mentioned that the processes are rather well understood macroscopically[4],

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i.e., ignition behavior in terms of the gross physical properties of the material and the parameters of exposures. Less well understood, however, are the details of the high temperature pyrolysis reactions and the role of changes of composition of the resulting vapors in the spontaneous ignition process.

In the analytical procedure to be described here, the vapors resulting under various conditions of pyrolysis are characterized through the use of two different systems. The first separates the condensibles into rough fractions with dynamic cold traps and analyzes the non-condensibles with suitable solid adsorption chromatography columns. Subsequently, the materials condensed in the cold traps are analyzed by liquid partition methods.

APPARATUS AND PROCEDURES

Exposure equipment

The work to date has been restricted to the study of rapid pyrolysis of α-cellulose in thin sheet form. To simplify the geometry of temperature distribution and, hence, provide a situation which is amenable to heat transfer theory, the material is suspended in such a way that it may be irradiated uniformly over one surface with small conductive loss. This is achieved by cutting the material into a circular wafer whose diameter is no larger than the diameter of the field of uniform irradiance provided by the source of radiation (generally less than 1-in. diameter) and suspending the wafer behind a quartz window in the center of the carrier gas stream with three tungsten needles which pierce the material near its edge (Fig. 1). Liquids, crystalline solids or any substances which cannot be readily produced in sheet form could be handled by saturating thin asbestos or quartz fiber paper with the substance and handling as described above. It is very important in the design of the exposure chamber, as it is in the flow system which follows, that the vapors be moved rapidly and uniformly with little or no "hold up". The resolution of the resulting chromatogram depends to a large extent upon the mixed vapors moving onto the column in a short, sharply-defined interval of time. Moreover, the vapors which arise from the irradiated solid attenuate the incoming radiation and, for this reason, should not be allowed to accumulate between the exposed surface and the quartz window. This requirement necessitated high helium flow rates through the system including the chromatography column. Inasmuch as the exposure chamber cannot be made smaller than about 15 ml capacity, it is necessary to run the carrier gas through at a rate of not less than 150 ml/min in order to carry away the expelled vapors at a velocity in accord with their free-air convection velocity. Since it is also important not to produce excessive forced convective cooling of the irradiated solid, in general the flow rate has been kept between 150 and 200 ml/min. To achieve this flow rate through a 2 or 3 m column packed with 30–60 mesh solid, it is necessary to use a larger-than-ordinary diameter copper tubing for the column (3/8-in. diameter), to avoid excessively high pressures in the exposure chamber and cold-trap train, and to use reduced pressures at the column exit. Generally, evacuating the exit to about 100 mm Hg pressure and supplying the helium at 5 p.s.i.g. provides the desired flow rate.

Cold traps

The cold traps are specially designed to provide turbulent gas flow along the full length of immersed tubing, the length of which was calculated to provide cooling of the emergent stream to approximately 90% of the bath temperature–ambient temperarature difference.

Cold trap media are selected according to the particular column packing material in use at the time. For example, the high-activity charcoal (Burrell 341-10) column at room temperature resolves the diatomic gases and methane, but ethane is irreversibly adsorbed. The equilibrium vapor pressure of ethane at 100° K is less than 0.1 mm Hg while methane and the diatomic gases, H_2, O_2, N_2 and CO have vapor pressures in the range of 1/4 atm and higher. Hence, liquid nitrogen appears to be a rather ideal cooling medium for the cold trap just preceding the high-activity charcoal column. Similar, though less clean-cut, separations are afforded by other cooling media, such

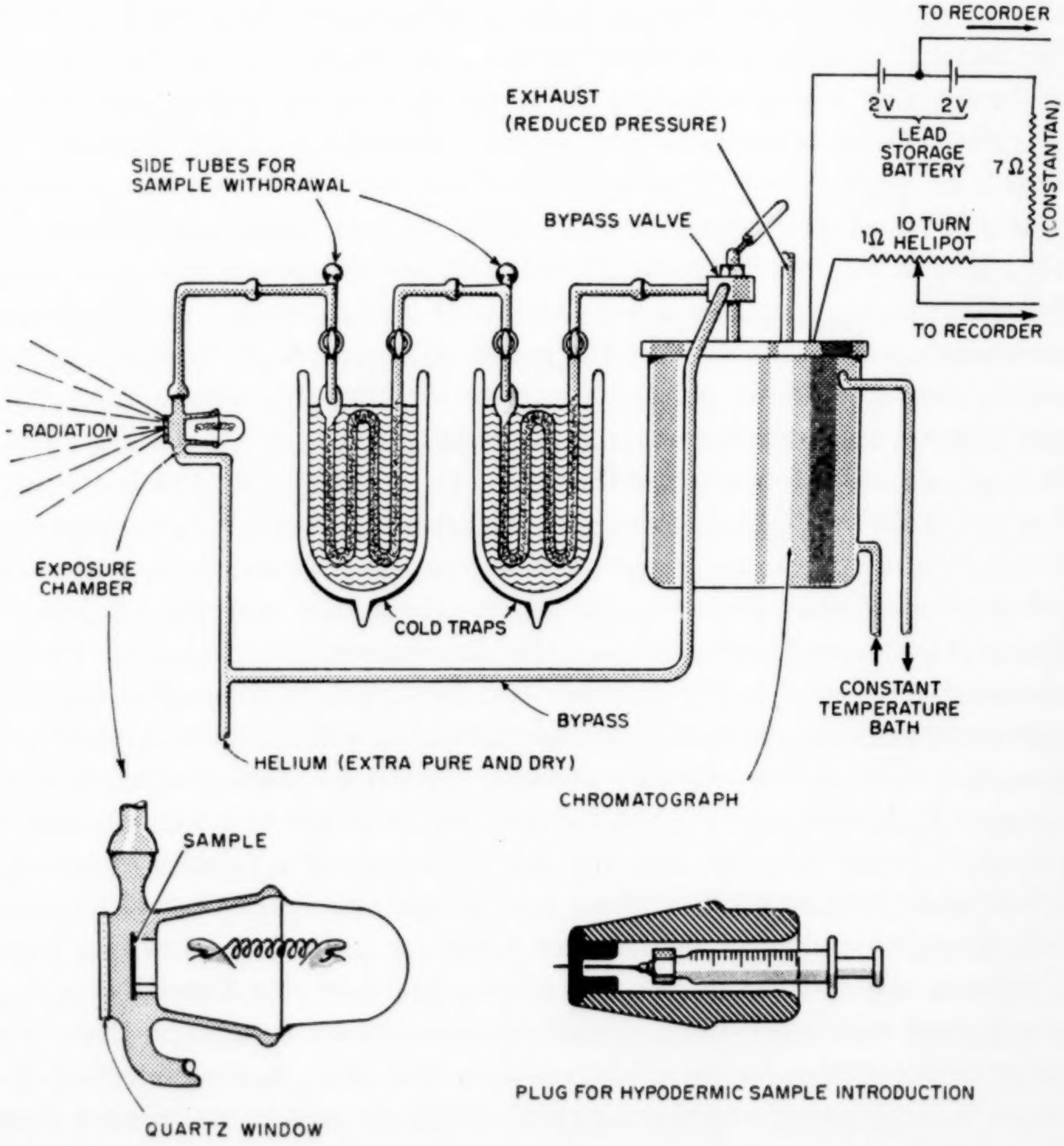
TO RECORDER
EXHAUST
(REDUCED PRESSURE)
2V 2V
LEAD
STORAGE
BATTERY
7 Ω
(CONSTANTAN)
SIDE TUBES FOR
SAMPLE WITHDRAWAL
BYPASS VALVE
1Ω
10 TURN
HELIPOT
TO RECORDER
- RADIATION
EXPOSURE
CHAMBER
COLD TRAPS
CONSTANT
TEMPERATURE
BATH
BYPASS
HELIUM (EXTRA PURE AND DRY)
CHROMATOGRAPH
SAMPLE
PLUG FOR HYPODERMIC SAMPLE INTRODUCTION
QUARTZ WINDOW

Fig. 1. Diagram of exposure apparatus and gas–solid adsorption chromatograph for the analysis of the non-condensed gases.

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as dry ice–acetone or dry ice–ether mixtures, ethanol slush (prepared by mixing the alcohol with liquid nitrogen), and ice–salt mixtures.

Some of the complications inherent in this type of separation should be mentioned. First of all, though an almost immeasurably small vapor pressure may exist for a particular substance at the temperatures achieved by cold trap cooling, the actual efficiency of the trap can never be better and generally will not be nearly as good as the efficiency calculated using the thermodynamic quantity "equilibrium vapor pressure" since the system is far from being at rest. Furthermore, the trap efficiency can never be 100% for any substance, for no matter how small the vapor pressure becomes, it is always finite at any real temperature. Secondly, materials which are supposed to "pass" the trap may be carried down and occluded with condensibles despite the fact that at the trap temperature their vapor pressure may exceed manyfold their partial pressure in the gas phase.

Recognizing these potential sources of error, the following steps have been taken to evaluate their effect and/or to obviate them:

1. A bypass for the exposure chamber and cold-trap train was provided so that at the conclusion of an exposure, as soon as the non-condensibles were on the column, the carrier gas could be diverted directly into the column entrance. In this way, any components of the condensate having appreciable vapor pressures were prevented from migrating out of the cold traps and onto the column.
2. The efficiency of the liquid nitrogen cold trap to retain ethane and ethylene was verified by analyzing the emerging gases with a silica gel column. Likewise the separations afforded with the ethanol slush and with dry ice–acetone were determined with alumina and dinonyl phthalate columns.
3. To test for occlusion the condensate in sealed-off traps was repeatedly vaporized and recondensed slowly, and the non-condensed vapors analyzed with the appropriate column packing.

Analysis of the non-condensible gases

Fig. 1 is a schematic diagram of the component parts of the chromatography unit designed to analyze the non-condensed fraction of the degradation products. Unlike most systems, there is only a single detector involved. The base line drift, frequently reported as an intolerable feature of single detector units, has not been found to reduce the general utility of the instrument, though at times it is somewhat annoying. The detector element was constructed by removing the envelope from a 6 V pilot light* and sealing the base, vacuum tight, into a 1/4-in. to 1/8-in. IPS reducer threaded into a massive brass block. The detector block along with the column is immersed in water whose temperature is maintained constant and kept stirred through the agency of an external constant temperature bath. The detector element forms one arm of a simple, yet very stable, bridge circuit (Fig. 1). The unbalance of the bridge, produced by the change in temperature of the tungsten filament as the heat transfer property

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* General Electric No. 44.

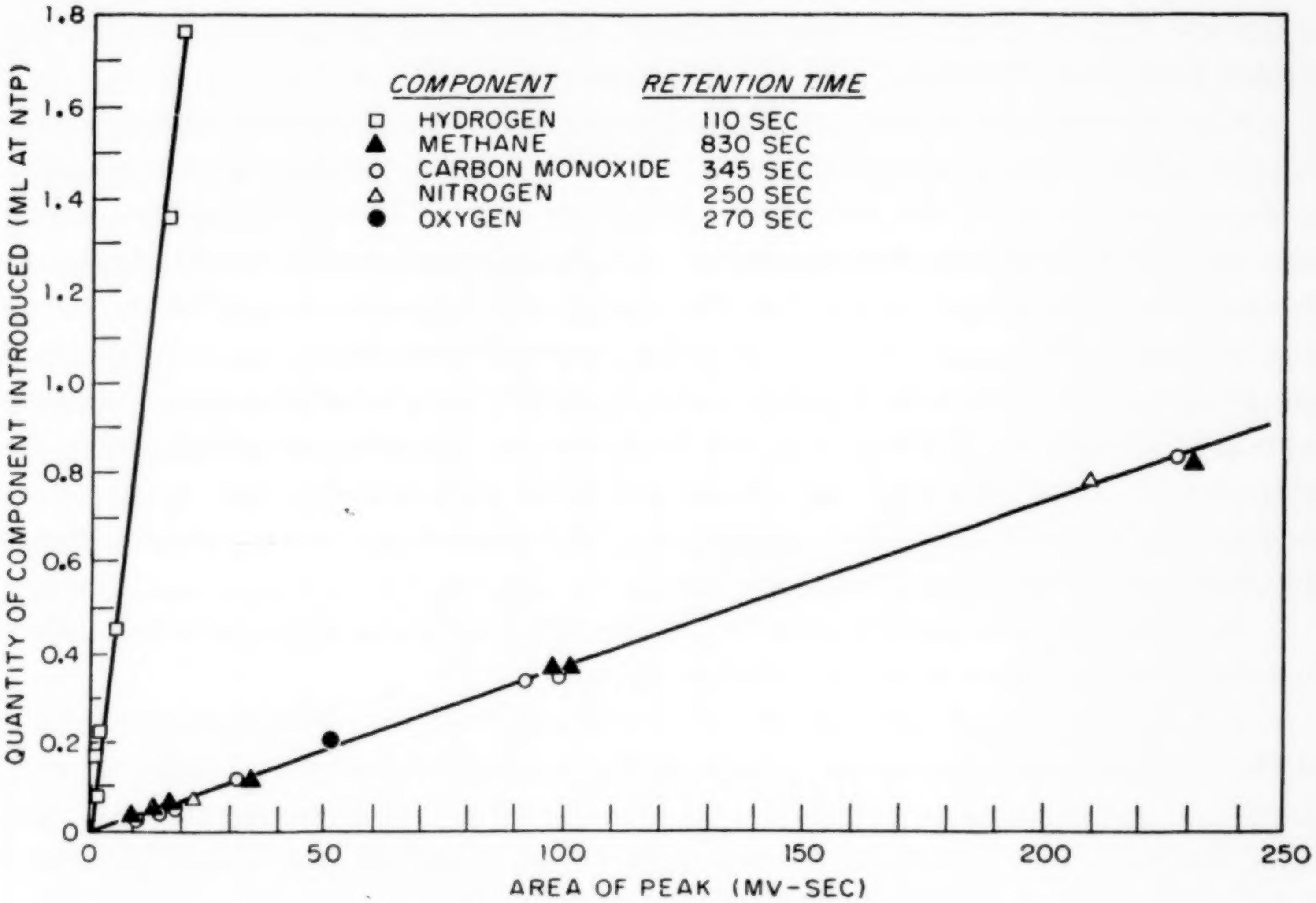

Fig. 2. Calibration of 3-m charcoal column.

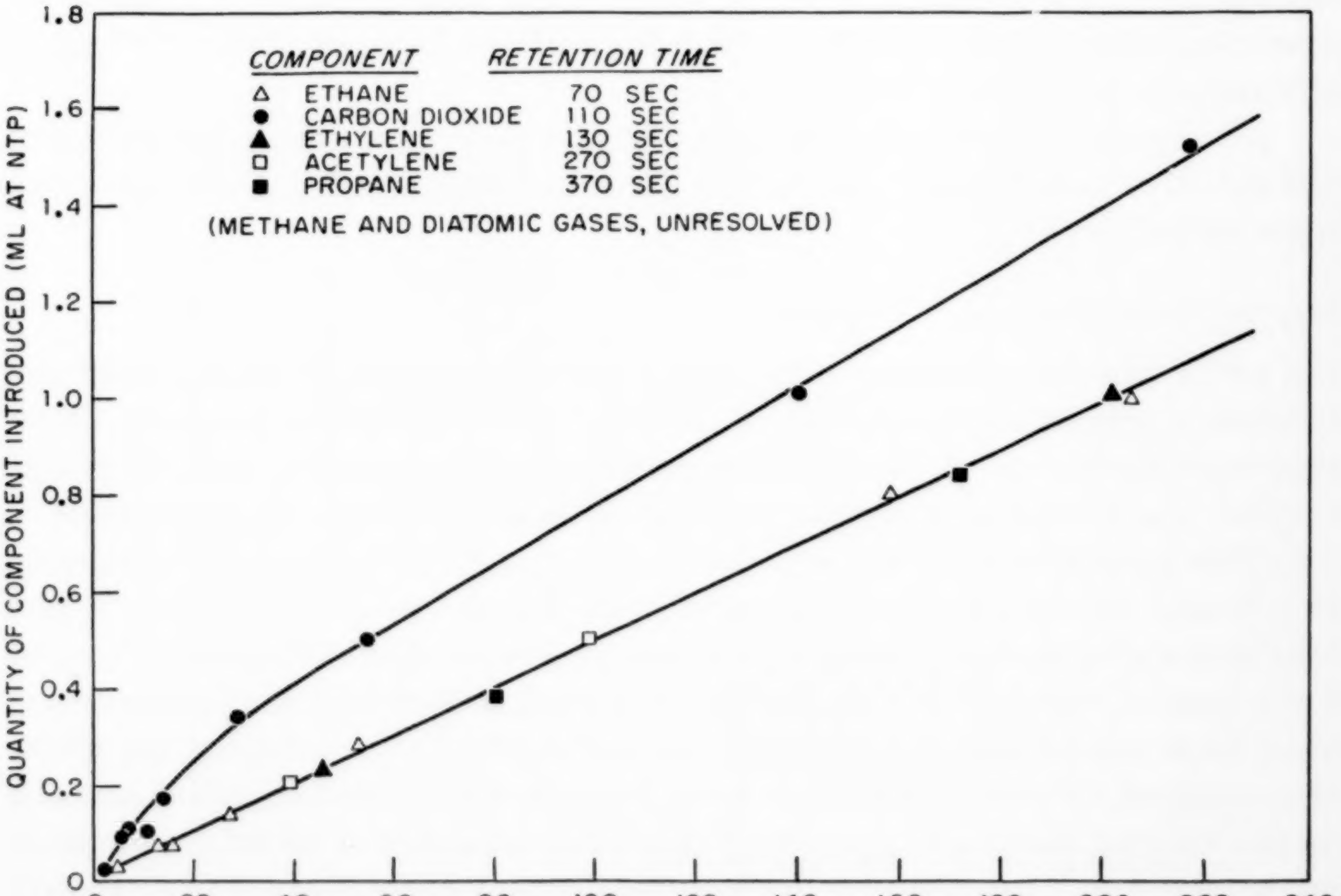

Fig. 3. Calibration of 2-m silica gel column.

of the gases in which it is immersed changes with changes in composition, is recorded on a chart-type potentiometer recorder having 0.5, 2.5, 10 and 50 mV full-scale sensitivities.

The system is calibrated by introducing with a hypodermic syringe through a special fitting in the exposure chamber (see Fig. 1) measured quantities of either pure gases or gas mixtures whose composition has been measured with a mass spectrometer. Example calibration curves are given in Figs. 2 and 3.

Table I lists the combinations of chromatography columns and cold trap media presently in use along with a brief description of the separations afforded by each. It

TABLE I

COMBINATIONS OF COLD TRAPS AND COLUMNS

Cold trap–column combinations *a. Higher temp. trap* *b. Lower temp. trap* *c. Column packing*	*Materials retained by cold traps*	*Analysis provided by column*	*Comments*
a. Dry ice–acetone b. Liquid nitrogen c. High-activity charcoal	a. Hydrocarbons except C_2 and C_3 and most oxygenated compounds b. C_2 and C_3 hydrocarbons plus CO_2 and ketene	H_2, N_2, O_2, CO, CH_4	Contents of trap a. analyzed by aliphatic phthalate stationary phase and with polyglycol stationary phase columns. Trap b. with silica gel column as well as with a polyglycol column as a check for the highly volatile oxygenated compounds, *e.g.*, CH_2O, CH_2CO
a. Dry ice–acetone b. Liquid nitrogen c. Silica gel	Same as above	(H_2,N_2,O_2,CO)*, CH_4 C_2H_6, C_2H_4,C_2H_2, CO_2 and C_3 hydrocarbons	Same as above. Used as a check on efficiency of liquid nitrogen to stop C_2 hydrocarbons
a. Sodium chloride–ice b. Dry ice–acetone (or ethanol slush) c. Silica gel	a. All normal liquids b. C_4 hydrocarbons plus volatile oxygenated compounds including CH_2O, CH_3CHO, and CH_3OCH_3	Same as above	Both traps analyzed as trap a. above
a. Sodium chloride–ice b. Dry ice–acetone (or ethanol slush) c. Dinonyl phthalate on C-22	Same as above	General for aliphatic compounds above C_2 or C_3 and for many oxygenated compounds, particularly esters and ketones	Used only to check efficiency of cold traps to stop intermediate range of compounds
a. Sodium chloride–ice b. Dry ice–acetone (or ethanol slush) c. Activated alumina	Same as above	Same as above	Same as above

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* Not resolved.

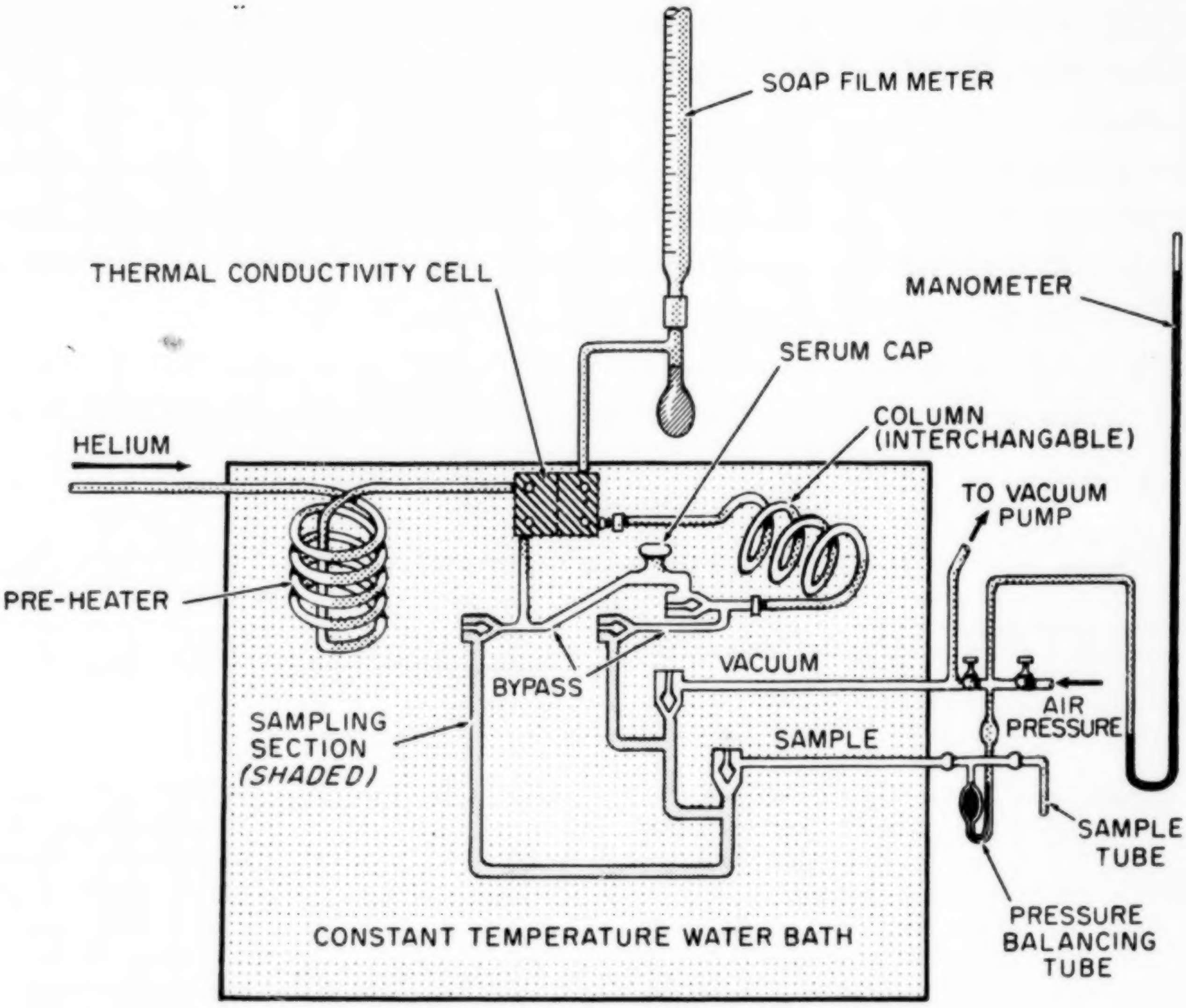
SOAP FILM METER
THERMAL CONDUCTIVITY CELL
MANOMETER
SERUM CAP
COLUMN (INTERCHANGABLE)
HELIUM
TO VACUUM PUMP
PRE-HEATER
VACUUM
BYPASS
AIR PRESSURE
SAMPLING SECTION (SHADED)
SAMPLE
SAMPLE TUBE
PRESSURE BALANCING TUBE
CONSTANT TEMPERATURE WATER BATH

Fig. 4. Schematic diagram of gas–liquid partition chromatography apparatus.

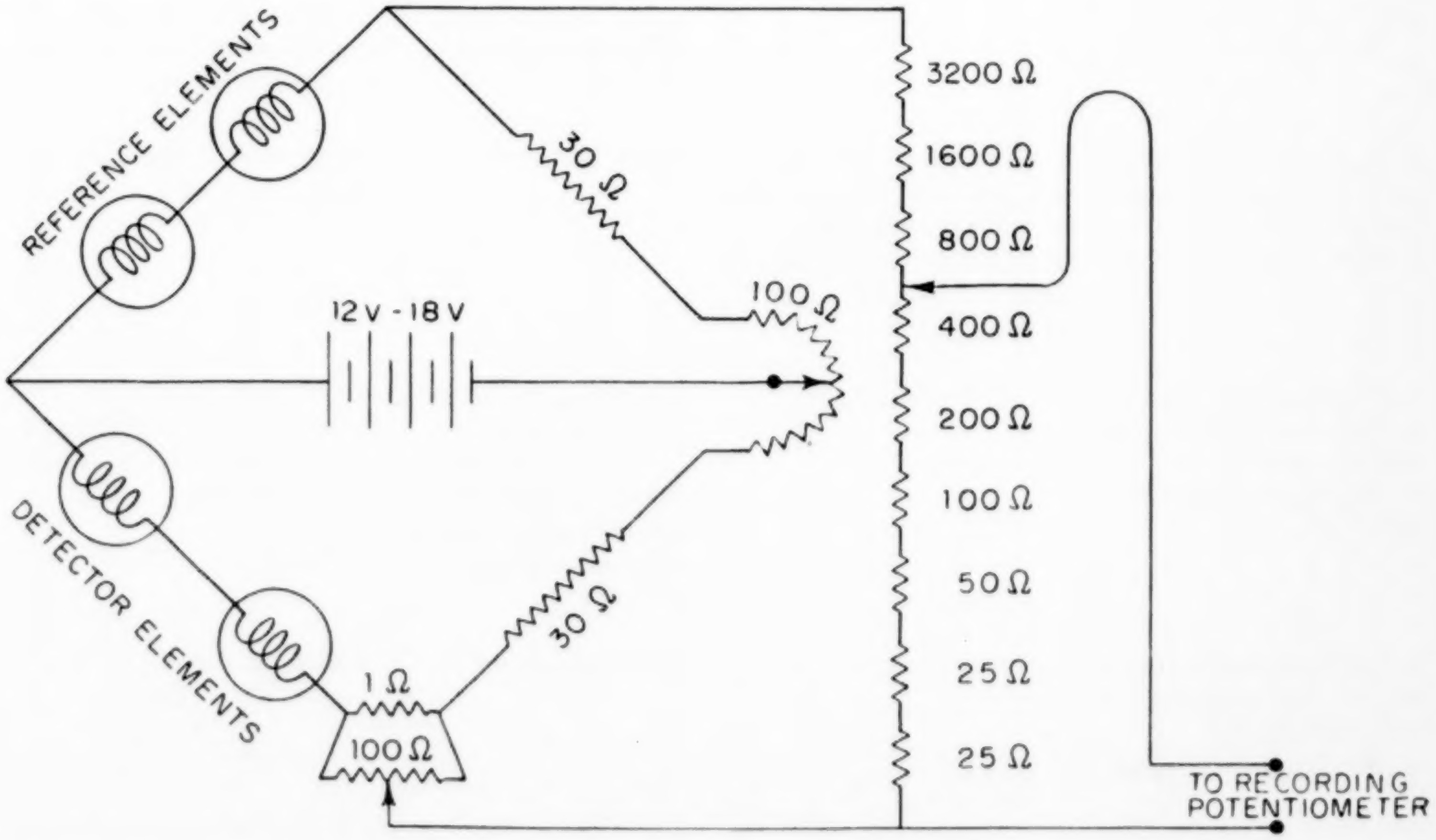
REFERENCE ELEMENTS
30 Ω
3200 Ω
1600 Ω
800 Ω
100 Ω
12 V - 18 V
400 Ω
200 Ω
100 Ω
50 Ω
DETECTOR ELEMENTS
30 Ω
25 Ω
1 Ω
100 Ω
25 Ω
TO RECORDING POTENTIOMETER

Fig. 5. Schematic diagram of bridge circuit for the gas–liquid partition chromatography system.

will be noticed that there is some overlap of utility. This provides a valuable check of one on another. The columns most often used are the high-activity charcoal and the silica gel. It is much more convenient to analyze the higher molecular weight materials in a more conventional chromatograph.

Analysis of condensed materials

The chromatography equipment used to analyze the mixtures of substances condensed in the various cold traps is essentially that which was reported by DIMBAT, PORTER

TABLE II

ELUTION DATA FOR TYPICAL COMPOUNDS IN THREE TYPES OF COLUMNS

	Vacuum pump oil 1-m column *at 40°* *Inlet pres.,* $P_i = 1.2$ *atm* *Exit pres.,* $P_0 = 1$ *atm* *Flow rate,* $f = 0.83$ *ml/sec* *Volume of liquid phase* $v_l = 4.9$ *ml*			*Dinonyl phthalate 2-m column* *at 70°* $P_i = 1.7$ *atm* $P_0 = 1$ *atm* $f = 0.83$ *ml/sec* $v_l = 4.7$ *ml*			*Polyethylene glycol 2-m column* *at 70°* $P_i = 1.5$ *atm* $P_0 = 1$ *atm* $f = 0.85$ *ml/sec* $v_l = 3.7$ *ml*		
Compound	*t_e *(sec)*	**v_e *(ml)*	***h	t_e	v_e	h	t_e	v_e	h
Ethane				12	7	1			
Propane				38	22	5			
Isobutane				70	41	8	20	13	3
n-Butane				105	62	13	30	20	5
2,2-Dimethyl-butane	350	265	132	355	210	43	125	82	21
Isopentane	125	95	48	205	121	25	55	36	9
n-Pentane	165	125	64	255	151	31	70	46	12
2-Methylpentane				485	287	59	155	101	26
3-Methylpentane				530	314	64	155	101	26
n-Hexane	490	371	186	645	382	78	155	101	26
n-Heptane	1450	1099	550						
Dimethyl ether	35	27	14	85	50	10	35	23	6
Diethyl ether	185	140	70	350	207	43	130	85	22
Dipropyl ether	1020	773	386	1510	894	184	520	340	89
Isopropyl ether	420	318	159	705	417	86	245	160	42
Methanol	80	61	30	320	189	39	520	340	89
Ethanol	135	102	51	600	355	73	850	555	145
n-Propanol	520	394	197	1510	894	184	1860	1215	317
2-Propanol				820	485	100	850	555	145
2-Butanol				1940	1148	236	1940	1267	330
2-Methyl-2-propanol							850	555	145
Acetaldehyde	40	30	15	195	115	24	100	65	17
Propionaldehyde	110	83	42	520	308	63	235	153	40
Acetone	120	91	46	605	358	74	315	206	54
2-Butanone				1250	740	152	670	438	114
3-Methyl-2-butanone				2200	1302	267	1000	653	170
3-Pentanone				3400	2013	413	1250	816	213
Methyl formate				215	127	26	125	82	21
Ethyl formate				590	349	72	300	196	51
Ethyl acetate				1250	740	152	600	392	102
Water				430	255	52	1140	744	194
Benzene	810	614	307	1850	1095	225	780	509	133
Toluene	2640	2001	1000	4700	2782	571	1700	1110	289

* Elution times, t_e, measured from air peaks.

** Elution volumes, $v_e = 3/2\ t_e f \dfrac{(P_1/P_0)^2 - 1}{(P_1/P_0)^3 - 1}$.

*** Partition coefficient, $h = v_e/v_l$.

References p. 283.

AND STROSS[5] except that constant temperature environment has been provided by building the system into a large constant temperature water bath (Labline No. 3052).

The chromatograph is provided with a serum-cap sample-introduction port, but is designed primarily for the analysis of vaporized samples introduced from an internal, constant volume sampling section which can be bypassed by the carrier gas stream while it is being evacuated and filled. The chromatograph may be operated in the temperature range 10° to 90° with a high degree of temperature control and constancy. See schematic diagram, Fig. 4.

Because of the high-temperature limitation of the water, this system does not permit the analysis of tarry materials which are formed in greater proportions in exposures of the lower levels of radiant power.

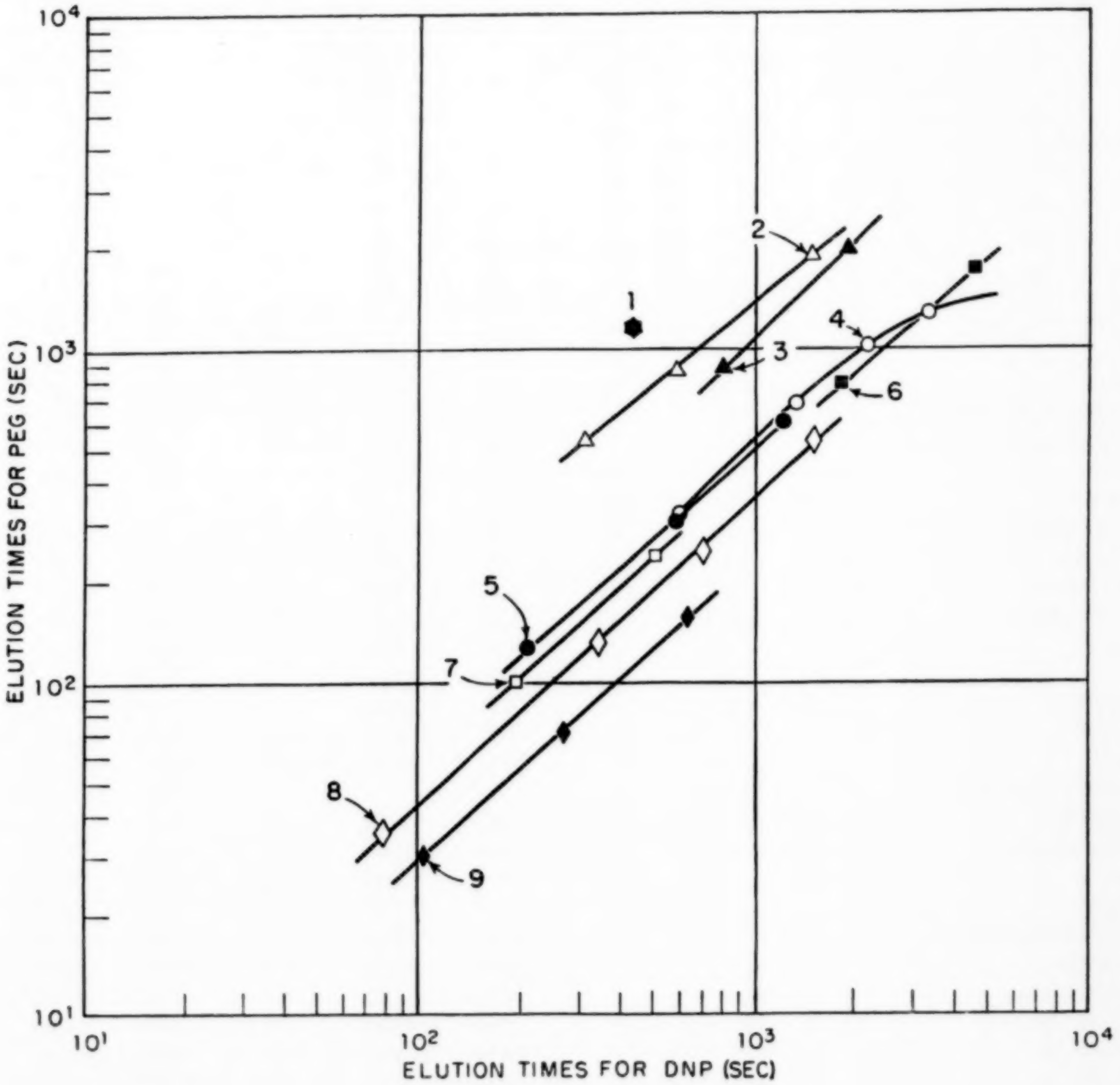

Fig. 6. Relative elution times for polyethylene glycol (PEG) and dinonyl phthalate (DNP) columns. 1. Water. 2. Primary alcohols: methanol, ethanol and *n*-propanol. 3. Secondary alcohols: isopropanol and *sec.*-butanol. 4. Ketones: acetone, 2-butanone, 3-methyl-2-butanone and 3-pentanone. 5. Esters: methyl formate, ethyl formate and ethyl acetate. 6. Aromatic hydrocarbons: benzene and toluene. 7. Aldehydes: acetaldehyde and propionaldehyde. 8. Symmetric ethers: methyl ether, ethyl ether, isopropyl ether and propyl ether. 9. Alkanes: *n*-butane, *n*-pentane and *n*-hexane.

Interchangeable columns of 1/4-in. O.D. copper tubing of lengths ranging from less than 1 m to greater than 20 m in length may be used. The solid support packing is crushed C-22 firebrick (Johns Manville) wet-sieved with running water to 40–60 mesh. Many different stationary liquid phases have been used including dinonyl phthalate, polyethylene glycol, dimethyl sulfolane, vacuum pump oil, and tetraethylene glycol dimethyl ether. In general, two meter columns of the first two of these packings are capable of resolving the composition of the condensate from cellulose pyrolysis.

The carrier gas is helium whose flow rate is kept at 50 ml (N.T.P.)/min. The flow rate is determined by the soap film method as described by JAMES[6].

The detector is a commercially available thermal conductivity cell (TE-II geometry, 4 GT-T filament, GOW-MAC Instrument Co., 100 Kings Road, Madison, N.J.) which was potted in apiezon hard wax to obviate possible effects of continued submersion in water. The electrical circuitry is shown in Fig. 5.

Elution data and h values corrected for the pressure drop across the column[7] are listed in Table II for three of the columns used, a 1-m column with vacuum pump oil (Cenco Hyvac) at 40° and two 2-m columns of dinonyl phthalate (Morton-Withers Morflex 190 DNP) and polyethylene glycol (Dow Polyglycol P-400) both at 70°. Following the suggestion of LEWIS, PATTON AND KAYE[8] on the use of two columns of different characteristics as a convenient means of qualitative analysis, the elution time values for the dinonyl phthalate and polyethylene glycol columns have been plotted on log-log paper in Fig. 6 to indicate the separations afforded.

Transfer of samples

At the conclusion of an exposure, after the cold traps have been bypassed and individually isolated, their contents are transferred to small volume sample tubes which are kept at liquid nitrogen temperature until ready for analysis. Because the total condensate in any one cold trap may be as small as 10 mg and may contain a dozen or more components, it is quite important that extreme care be exercised in the handling of the sample to prevent any loss or contamination. Also it is essential to keep the volume of the sample introduction and associated pressure measuring equipment small by comparison to the volume of the actual sample introduced. The sequence of operations is depicted in Fig. 7. After the cold trap has been isolated, the sample tube is connected and communicated through the three-way stop cock of the cold trap to a vacuum pump. After the tube has been thoroughly outgassed, it is communicated to the trap section, immersed in liquid nitrogen and the trap is allowed to warm up until the condensate has been entirely transferred to the sample tube. The sample tube stop cock is then closed and the tube is transferred to the chromatograph where it is communicated to the sampling section of the chromatograph through a small-volume pressure balancing tube. The entire sampling system is then evacuated and the sample vaporized completely by warming the sample tube, maintaining the mercury level in the pressure balancing tube by continually increasing the opposing pressure. Once at equilibrium, the pressure in the sampling system is ascertained by reading the

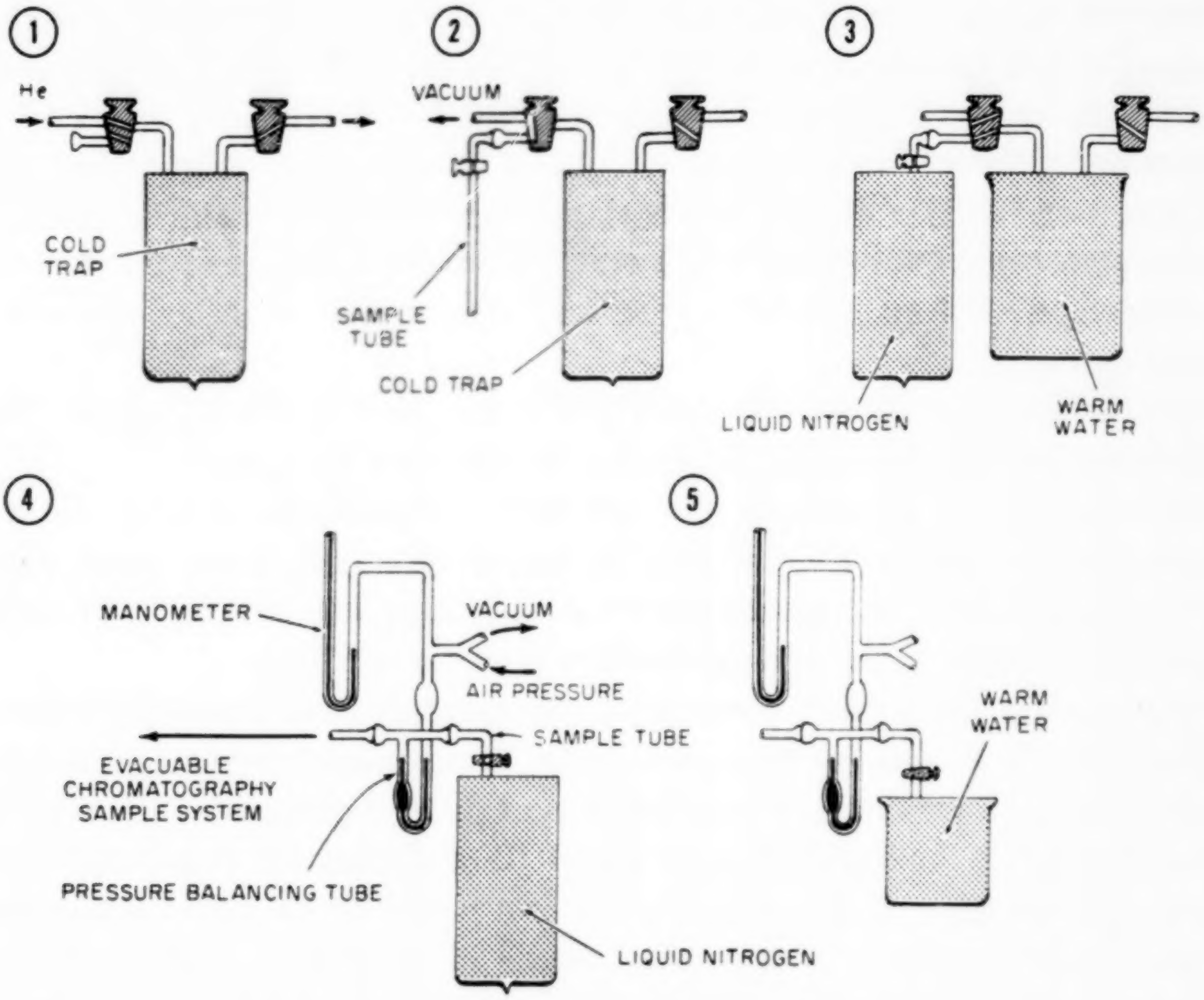
①
He
COLD TRAP
②
VACUUM
SAMPLE TUBE
COLD TRAP
③
LIQUID NITROGEN
WARM WATER
④
MANOMETER
VACUUM
AIR PRESSURE
SAMPLE TUBE
EVACUABLE CHROMATOGRAPHY SAMPLE SYSTEM
PRESSURE BALANCING TUBE
LIQUID NITROGEN
⑤
WARM WATER

Fig. 7. Series of schematic drawings depicting sequence of operations in transfer of sample from cold trap to chromatograph.

opposing external pressure which just balances the mercury levels. Knowing the volume of the sample section of the chromatograph and its temperature, the total quantity of sample can be readily calculated. The sample is then isolated and swept onto the column and analyzed.

DISCUSSION OF RESULTS AND CONCLUSIONS

The methods described have proven to be quite satisfactory in their present application, *i.e.*, investigation of the reaction products of the pyrolysis of cellulosic solids irradiated by intense radiant energy. Products of low molecular weight (up to about 50) can be analyzed directly by a chromatograph incorporated into the exposure equipment. This chromatograph, generally utilizing gas–solid adsorption chromatography, is designed to quantitatively determine the composition of the gases which are not condensed by the cold traps which precede it. The possibility of overlooking a substance which is not retained (or of underestimating the amount of a substance which is only partially retained) by the cold trap and for which the column packing is not a suitable partitioning medium is obviated by repeating the experiment using different combinations of column packing and cold trap media. This procedure also provides a valuable check of the reproducibility of the experimental results and a means for evaluating comparisons of the quantity of substances which appear in the chromatograms of different columns by the comparison of the amounts of a single substance which appear in the chromatogram of both columns.

This apparatus when used in connection with the conventional chromatograph, permits the complete separation of all of the many products occurring in the evolved vapors of cellulose pyrolysis.

SUMMARY

Gas chromatographic methods are described for determining the products evolved by organic solids (or liquid with small vapor pressure) when rapidly decomposed directly in the carrier gas stream by suitable external stimuli. The method, presently employed to study the chemical reactions accompanying the ignition of cellulosic solids by intense radiant energy, utilizes special dynamic cold traps to separate the complex products into fractions which can be conveniently analyzed by the various gas–solid adsorption and gas–liquid partition columns.

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

PART IV. THE SEPARATION AND DETERMINATION OF SOME HEAVY METALS IN ADMIXTURE WITH LEAD*

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Although many paper chromatographic procedures[1-11] have been published for the separation of lead from other heavy metals, these have been mainly concerned with the qualitative separation of the Groups I and IIA of the general qualitative analytical scheme. Such separations, although invaluable for qualitative purposes, are unsuited for quantitative chromatography where complete separations are usually required. LEWIS AND GRIFFITHS[12] showed how problems in polarographic analysis were overcome by paper chromatography. The eluting solvent employed was 5% v/v concentrated hydrochloric acid (1.18) in *n*-butanol; after elution and extraction the lead was estimated polarographically in the presence of vanadium, copper, uranium, and titanium. Unfortunately the polarographic method used was of low sensitivity and for accurate results great care was needed. For the estimation of lead in natural waters, CARRITT[13] passed the sample down a column composed of a solution of diphenylthiocarbazone in carbon tetrachloride absorbed on cellulose acetate. Heavy metals were absorbed as their respective dithizonates. The column was then stripped of cadmium, zinc, manganese, and lead by treatment with 1 *M* hydrochloric acid and these metals were simultaneously estimated polarographically. HUNT, NORTH AND WELLS[14], separated other metals from lead using paper strips and eluting with 5% v/v concentrated hydrochloric acid (1.18) in methanol. The method was applied to the semi-quantitative estimation of lead in soils.

The work to be described was undertaken in an attempt to resolve simultaneously the following analytical problems:

(1) The lengthy procedure necessary for the isolation and accurate determination at μg level of more than one heavy metal ion on the same sample of solution[15-18]. In general the published analytical procedures show a number of methods but most of these are designed for the estimation of one element per aliquot of sample solution taken. Thus when a mineral analysis for four or five elements is required, the time taken to complete such a task is prohibitive, and often only a small quantity of material is available[19].

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[*] For parts I, II and III, see ref. [23,24,27] respectively.

References p. 295.

(2) The complications and errors incurred when employing diphenylthiocarbazone for the extraction and absorptiometric estimation of lead[20, 21].

(3) The lack of data concerning the quantitative chromatographic behaviour of the lead ion.

APPARATUS AND GENERAL TECHNIQUE

The downward elution method was used, the apparatus for which has been described previously[22–24]. The all-glass apparatus consisted of two troughs (24 cm long by 2.5 cm in diameter) supported on a glass stand contained in a tank (23 cm × 28 cm and 52 cm deep) the top of which was smeared with grease, and covered with a ground-glass plate drilled with two holes located one over each trough. The purpose of these holes was to permit solvent to be admitted into the trough without disturbing the equilibration of the tank. The solvent mixture used for equilibrating the tank and for elution had the same composition.

The Whatman No. 1 filter-paper used for the chromatographic separation was purified by acid washing for six days and then washed free from chloride with distilled water[23, 25]. The paper chromatogram bearing the metals to be separated was placed in position in one of the troughs, solvent was poured into the other trough and into the bottom of the tank, the apparatus being left to equilibrate overnight. Solvent was poured into the trough containing the chromatogram through the hole in the cover plate and the solvent allowed to percolate 35 cm down the paper. The chromatogram was then removed, dried and sprayed.

The eluting solvent

In quantitative chromatography it is necessary to use metal salts corresponding to the acid species in the solvent in order to prevent the metal ions from forming more than one spot[26]. The salts chosen in this case were the metal nitrates since all of them (where nitrates exist) have the common property of being soluble in water. Consequently if the eluting solvent contains dilute nitric acid, true "partition" chromatography may occur and not precipitation chromatography, *i.e.* the precipitation of an insoluble salt near the starting line due to the presence in the solvent of an anion of an acid capable of forming such a salt with the metal ion under investigation. In many such cases the insoluble salt streaks forward due to the slight solubility of the precipitated salt in the solvent as it percolates over the band. This causes low recovery values to be obtained unless special precautions are taken.

The solvent system for the separation of lead, zinc, and mercuric mercury on acid-washed Whatman No. 1 filter-paper was developed by A. J. BANISTER of these laboratories, employing the semi-graphical technique and ascending elution[27]. The solvent A finally selected, consisted of 50 ml diethyl ether, 30 ml methanol, 20 ml water, and 2 ml of AnalaR nitric acid (1.42). To determine the R_F values of the heavy metal ions under consideration in this solvent but employing descending elution, a series of chromatograms were eluted using single spots of the aqueous metal nitrates (each spot contained approx. 15 μg of the metal). The chromatograms

TABLE I

Metal ion	*Spray*	*Colour of spot*	*R_F value (head and tail of spot)*
Tl(I)	0.5% w/v 8-hydroxyquinoline in absolute alcohol. Hold over ammonia and u.v. light.	Yellow-green fluorescence	0.25–0.17
Ag(I)	5% w/v tannic acid in warm 60% v/v aqueous methylated spirits. Warm the damp strip.	Brown stain	0.36–0.27 Dark hydrolysis products also present at starting line
Pb(II)	Aqueous solution of rhodizonic acid and held over ammonia.	Red	0.41–0.22
Zn(II)	0.5% w/v 8-hydroxyquinoline in absolute alcohol. Hold over ammonia and under u.v. light.	Yellow fluorescence	0.66–0.55
Cd(II)		Yellow fluorescence	0.66–0.55
Cu(II)	0.1% w/v rubeanic acid in absolute alcohol. Hold over ammonia.	Green	0.66–0.56
Co(II)		Yellow-brown	0.68–0.55
Ni(II)		Blue	0.68–0.55
Mn(II)	4% v/v salicylaldehyde in 50% aqueous ethyl alcohol. Hold over ammonia and under u.v. light.	Dark spot against a yellow-green fluorescent background	0.70–0.56
Fe(II)	0.5% w/v aqueous potassium ferrocyanide.	Blue	0.70–0.58
Fe(III)		Blue	0.70–0.58
Bi(III)*	10% w/v freshly prepared aqueous sodium dithonite. Warm.	Brown	Hydrolysed all down the paper to 0.8
U(VI)	0.5% w/v aqueous potassium ferrocyanide.	Brown	0.78–0.72
Hg(II)	0.05% w/v dithizone in chloroform.	Pale pink	0.95–0.87

* Bismuth may be chromatographed successfully only if the original solution contained 5% v/v aqueous nitric acid, then no hydrolysis products are formed, and the R_F value of Bi is 0.78–0.67.

after elution were cut up and each strip sprayed with a sensitive reagent for the detection of the respective metal ion. Table I shows the R_F values of the metal ions chromatographed and the spray reagents employed for their detection. From Fig. 1 it may be seen that it is possible to separate completely the heavy metals of major interest (*i.e.* Zn(II), Cd(II), Cu(II), Fe(II), Ni(II), Bi(III), Hg(II)) from lead (Pb(II)) but the complete separation of lead, thallous thallium, and silver is impossible. Bismuth is only eluted satisfactorily when the applied spot contains 5% v/v nitric acid, but unfortunately ferric iron and bismuth overlap, if applied simultaneously to the chromatogram. A further investigation was then carried out to overcome this difficulty, and the solvent B selected was 50 ml diethyl ether, 30 ml methanol, 22 ml water and 4 ml of AnalaR nitric acid (1.42). (See Fig. 2.) When bismuth is present, solvent B must be used but for all other purposes solvent A is satisfactory.

Purity of materials

In the preliminary work on the estimation of lead, considerable difficulty was encountered due to the presence of lead, and other trace heavy metals in materials and apparatus used.

(*a*) *Apparatus.* Trace metals including lead are readily removed from porcelain or silica apparatus by boiling acids, so extraction techniques were developed which used only Pyrex glass vessels. The danger of "pick-up" was thus minimised and all apparatus was cleaned out with hot 10% caustic soda solution, followed by hot 1.1 aqueous nitric acid and copious washings with distilled water.

(*b*) *Water.* Water from a single distillation using a metal still was found to contain a fairly high percentage of heavy metals, usually of the order of 0.1 mg per litre. A double distillation from a steamed out all-glass (Pyrex) apparatus and storage in Jena glass vessels reduced this figure to approximately 0.01 mg per litre.

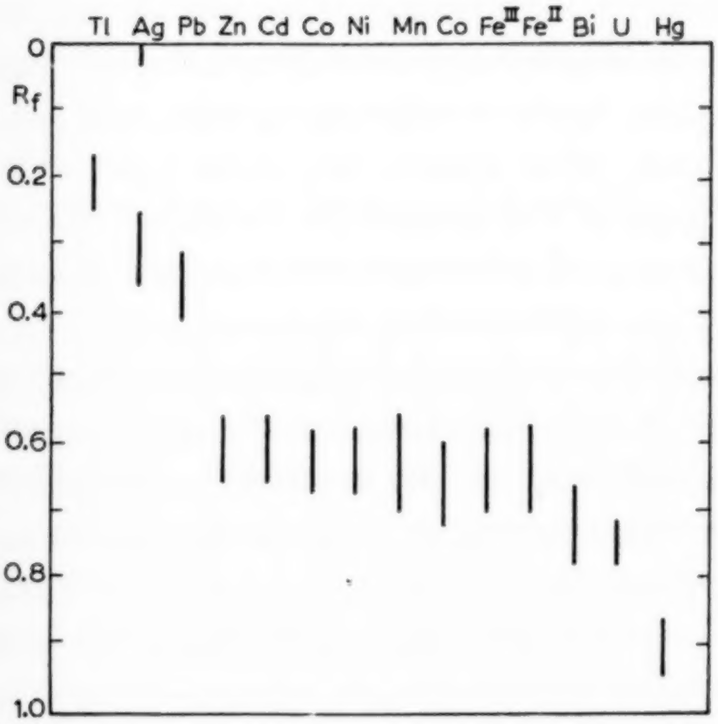


Fig. 1. The R_F values of 14 cations in descending elution with the solvent system Et_2O–MeOH–H_2O–HNO_3; 50:30:20:2 (Solvent A).

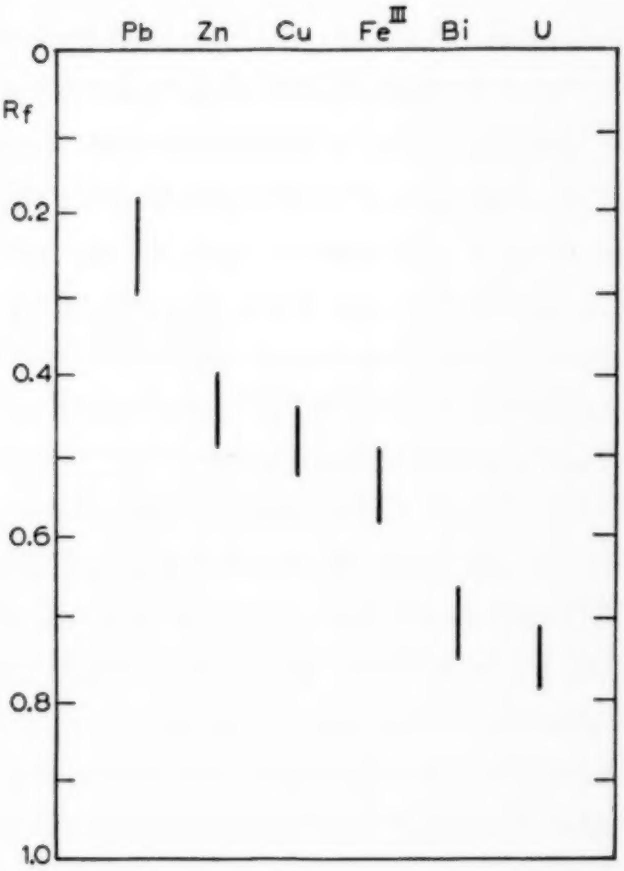


Fig. 2. The R_F values of 6 cations in the solvent system Et_2O–MeOH–H_2O–HNO_3; 50:30:22:4 (Solvent B).

(*c*) *Components of the solvent.* The organic components of the solvent were tested for their heavy metal content, using the dithizone method for lead[21] on the residue left after evaporation of the organic phase. The level of metal content was less than 0.05 μg of heavy metals per ml.

The lead content of the acid is relatively high, 2 μg of lead per ml, but the error involved was considered negligible since it is present only up to 4% by volume in the eluting solvent.

(*d*) *The filter-paper.* The problem of impurities in filter-paper has been discussed in some detail previously[23, 27] and will only be mentioned briefly. Iron is the major heavy metal impurity, the general content of Whatman No. 1 paper being 0.1 μg of iron per cm^2, this value may be decreased to 0.02 μg per cm^2 by acid washing but complete removal of iron is extremely difficult except by such treatment that destroys the fibrous nature of the paper. Many procedures were studied for the complete removal of iron but it appears that the solvent continuously dissolves away very small quantities of the cellulose during elution and thus sets free metallic impurities not previously removed in the washing procedure.

Great care must be taken over the storage of filter paper since iron pick-up from dust of the atmosphere over a period of time occurs very rapidly indeed. The method of purification of the paper has been described above (p. 285).

Design of quantitative chromatogram

Two main types of quantitative chromatogram were again used[24].

(1) *The wide band strip*[28]. A sheet of paper 20 cm by 55 cm was employed with the starting line marked 10 cm from the upper narrow edge. The solution to be chromatographed was applied as a band 12 cm long by 1 cm wide along the starting line from a calibrated Agla micrometer syringe. Spots of solution (approx. 0.02 ml) were applied 3 cm from the edges of the band. After elution two strips 3 cm wide were cut (one from each edge of the chromatogram) and sprayed for the metals under investigation. The pilot strips were then used as guides for the metal-bearing sections of the main chromatogram.

(2) *Twin paper strips*. The chromatogram consists of twin strips each 4 cm wide and 50 cm long divided by a 0.5 cm slot[23,24]. Identical volumes of solution were delivered along the two sections of the starting line, the wet bands being no wider than 1.5 cm. For larger volumes, the solution was put on the chromatogram in successive 0.025 ml portions, the chromatograms being dried with an electric dryer between each addition. After elution, one strip was then sprayed to locate the ions while the other was used for the estimations.

The second type of chromatogram was favoured for quantitative estimations for the reasons discussed in detail elsewhere[24]. To concentrate a smaller quantity of one metal in the presence of a large amount of another metal which moves to a lower R_F value, a tapered twin-strip chromatogram was used. The strips were tapered from a width of 4 cm to a width of 2 cm at a distance of 20 cm from the starting line. The width is constant (2 cm) for the lower 30 cm of the strips. By this means the metals are concentrated into a smaller area of paper, for which the blank correction is consequently less.

Extraction of metals from the chromatogram

Many extraction procedures were investigated for each metal ion but in general only (1) complete wet digestion of the paper by the conventional means for destroying organic material[29], or (2) acid leaching at an elevated temperature[30], gave quantitative recovery values. The disadvantage of method (1) is the time required in order to obtain a white ash residue and only method (2) will be described in detail.

(*a*) *Lead.* The section of the chromatogram containing lead was cut up into small pieces, placed in a 100 ml Pyrex beaker, covered with 10 ml of 2 M nitric acid and the solution boiled for 1 min. The extract was decanted off and stored. The extraction procedure was then repeated employing (1) 10 ml of 1 M nitric acid and (2) 10 ml of distilled water. The three extracts were combined, filtered through a Jena No. 3 sintered glass crucible and finally evaporated down to very small bulk using surface heating.

(*b*) *Bismuth.* Bismuth may be extracted by a procedure similar to (a) except that perchloric acid is employed in place of nitric acid.

(*c*) *Cadmium, copper, iron, and zinc.* If only one of these ions is present in the mixture extraction using a procedure similar to (a) but employing hydrochloric acid instead of nitric acid results in quantitative recovery. If, however, two or more ions are present, they will be fully separated from lead but not from each other since their R_F values are similar (Table I). Extraction with dilute hydrochloric acid on the metal-bearing section of the paper still yields quantitative recovery values but for individual estimation of each element a further separation has to be carried out (by anion exchange chromatography).

Anion-exchange separation of cadmium, copper, iron, and zinc

KRAUS AND MOORE[31] succeeded in separating the transition elements (manganese to zinc) in hydrochloric acid solutions by anion-exchange chromatography employing Dowex-1 resin. The adsorbabilities of these elements differ sufficiently as a function of the hydrochloric acid concentration to permit their ready separation at the mg level of concentration. RUSH AND YOE[32] using the same type of resin reported a separation of copper(II) and iron(III) from zinc(II) at microgram level. Copper(II) and iron(III) are eluted by 1 M hydrochloric acid while zinc is strongly adsorbed, zinc is later eluted from the column with 0.005 M hydrochloric acid. HUNTER AND MILLER[33] separated zinc from numerous other elements by anion-exchange in dilute hydrochloric acid using Amberlite IRA–400, in the concentration range 0.1–5 mg of zinc. KALLMANN, STEELE AND CHU[34] reported a separation of zinc and cadmium in dilute hydrochloric acid–sodium chloride solutions using Dowex-1 resin from most of the elements associated with zinc or cadmium. Zinc was quantitatively separated from cadmium by elution with 2 M caustic soda containing 20 g of sodium chloride/l, the cadmium was later eluted from the column with 1 M nitric acid. Unfortunately, no details of a separation of Fe(III), Cu(II), Zn(II), and Cd(II) at mg level was found in the literature. Since the elution constants of these elements are already available[31, 34] only the practical details for the separation will be given.

Apparatus. The column consisted of a glass tube 15 cm long, 0.5 cm radius fitted with a 2 mm glass tap at the lower end, and packed to a depth of 10 cm with Amberlite IRA–400 resin of 150 mesh size. The eluting agent was passed through the column using a 50 cm head of liquid. The column was purified by passage of several hundred ml of 1 M nitric acid, followed by copious washings with distilled water. The resin was then saturated with 3 M hydrochloric acid in readiness for use.

Procedure. The acid extract was placed on the column in the conventional manner and allowed to adsorb. The column was first eluted with 3 *M* hydrochloric acid and all the copper(II) was contained in the first 25 ml of eluant. The eluting agent was then changed to 0.1 *M* hydrochloric acid and the second 25 ml of eluant contained all the ferric iron. By changing to 1 *M* caustic soda containing 20 g of sodium chloride per l the zinc was quantitatively eluted in the following 50 ml of eluant, finally cadmium was eluted employing 1 *M* nitric acid.

The interference of both bismuth and lead in this method[34] was not studied since they are removed in the initial paper chromatographic separation. When nickel, cobalt or manganese are also present, they are extracted with the elements iron, copper and zinc from the paper chromatogram, and are eluted from the resin in 3 *M* hydrochloric acid and so accompany the copper(II). In the spectrophotometric method used for the estimation of copper they cause no interference.

SPECTROPHOTOMETRIC ESTIMATION OF THE METAL CONTENT OF THE EXTRACTS

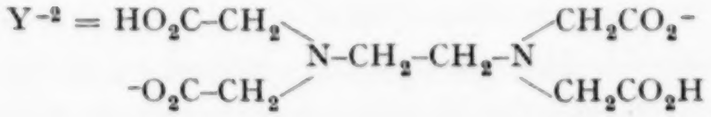
Lead

The residue after extraction and fuming was dissolved in 5 ml of 0.01 *M* nitric acid solution, and this solution was transferred to a 25 ml volumetric flask for absorptiometric determination. The concentration of acid is sufficiently low to cause no change in pH of the buffered solution in the absorptiometric procedure.

(*a*) *Eriochrome Black T procedure.* The most satisfactory reagents for absorptiometric analysis are those that yield a true aqueous solution of the metal complex. A search of the literature was made for such a reagent for lead, and the only suitable reagent found was Eriochrome Black T[35]. The latter is widely used in compleximetric titrations of Mg(II), Zn(II), Cd(II), Pb(II) and Hg(II) since it produces a reversible colour change when these metals are determined by ethylenediaminetetraacetic acid (EDTA). The reagent has also found use in the absorptiometric analysis of magnesium[36]. When an exactly analogous procedure for the estimation of lead to that described by Pollard and Martin was carried out, only a highly curved calibration graph was obtained. This signified that the complex was highly dissociated and the method was rejected.

In compleximetric titrations with EDTA, metals for which no suitable metal indicators are known may sometimes be estimated using the replacement technique. The metal (M) to be determined is transformed into the complex state by adding the pure complex of magnesium with EDTA. Where the EDTA complex of metal (M) is more stable than the Mg–EDTA complex, then an equivalent amount of magnesium ions are thereby produced.

$$M^{+2} + MgY \rightleftharpoons Mg^{+2} + MY$$



$$Y^{-2} = \begin{array}{l} HO_2C\text{–}CH_2 \\ {}^-O_2C\text{–}CH_2 \end{array}\!\!>N\text{–}CH_2\text{–}CH_2\text{–}N<\!\!\begin{array}{l} CH_2CO_2^- \\ CH_2CO_2H \end{array}$$

The magnesium ions released are then determined in the normal manner, *i.e.* by titration with EDTA. The purpose of the investigation was to see if it was possible to use this type of reaction and colorimetrically estimate the magnesium with Eriochrome Black T.

Employing the method due to POLLARD AND MARTIN[36] and adding 5.0 ml of 0.01 M magnesium–EDTA complexonate solution, a satisfactory method for the estimation of lead was developed. Beer's law is obeyed over the range 0–10 μg of lead per ml, and the slope of the calibration graph was 0.105 optical density units per μg of lead per ml at 520 mμ. Although this method is only one-third as sensitive as the dithizone method, it is preferred since it can be carried out in aqueous solutions. When copper, zinc, mercury, cadmium, or silver are present with lead, they may be effectively masked by the addition of potassium cyanide solution. The interference of iron and cobalt, however, cannot be eliminated since their cyanide complexes tend to oxidise Eriochrome Black T irreversibly when in strongly alkaline solution.

This method was used for the work on the binary and ternary mixtures of the heavy metals containing lead, but has recently been superseded by the method described below.

(*b*) *4-(2-Pyridylazo)-resorcinol.* This reagent has been synthesized in these laboratories and is especially noteworthy in that it forms a water-soluble chelate with lead salts which is suitable for spectrophotometric estimation of lead. Details of the synthesis and general properties of the reagent are described in a paper published elsewhere[37], and only details of the method for the estimation of lead will be described.

To the neutral sample containing between 0–125 μg of lead in a 25 ml volumetric flask are added 2.0 ml of 0.05% w/v aqueous 4-(2-pyridylazo)-resorcinol solution, 10 ml of buffer pH 10 (ammonium chloride in aqueous ammonium hydroxide solution) and the mixture diluted to 25 ml. The optical density of the solution is measured at 530 mμ against a similarly prepared blank solution containing no lead.

Beer's law is obeyed over the range 0–5 μg of lead per ml, and the slope of the calibration graph at 530 mμ is 0.165 optical density units per μg of lead per ml. This method was used for the lead analyses given in Tables II, III and IV.

Bismuth

After evaporation of the extract to a few ml, this solution was ready for absorptiometric analysis using the thiourea method described by LISICKI AND BOLTZ[38].

Iron

The boiled-down solution (or aliquot of the same) was transferred to a 25 ml volumetric flask. 2.0 ml of 5% w/v aqueous hydroxylamine hydrochloride solution was added, followed by 1 drop of 0.05% w/v aqueous methyl orange, and 0.5 M caustic soda solution until the indicator just turned yellow. Finally 1.0 ml of 0.247% w/v aqueous 2-nitroso-1-naphthol-4-sulphonic acid (sodium salt), together with 3.0 ml of 0.20 M acetic acid and 7.0 ml of 0.20 M sodium acetate solution were added. The solution was diluted to the correct volume and its optical density measured at 700 mμ against a similarly prepared reagent blank[27].

Beer's law is obeyed over the range 0–4 μg of iron/ml and the slope of the calibration graph is 0.428 optical density units per μg of iron per ml.

Copper

(*a*) *Biscylohexanone oxalyldihydrazone.* The boiled-down copper solution was transferred to a 25 ml volumetric flask and its copper content estimated by the procedure described by PETERSON AND BOLLIER[39].

(*b*) *Zincon.* The boiled-down copper solution was neutralised to approximately pH 5 employing methyl orange and 0.5 *M* caustic soda solution. The copper content was estimated employing the procedure described by RUSH AND YOE[32].

Zinc

(*a*) *Eriochrome Black T.* Zinc may be estimated by a procedure exactly similar to that described for the indirect estimation of lead employing Eriochrome Black T. The slope of the calibration graph is 0.335 optical density units per μg of zinc per ml at 520 mμ.

(*b*) *Zincon.* The boiled-down zinc solution was neutralised to about pH 9 by addition of 2 drops of 0.05% w/v neutral red solution, and 0.5 *M* hydrochloric acid until the indicator just changed from red to yellow. The procedure described by RUSH AND YOE[32] for the estimation of zinc using Zincon was employed.

Simultaneous estimation of copper and zinc with Zincon

If only copper and zinc are present then they may be estimated in the manner described by RUSH AND YOE[32]. Maximum absorption of the copper complex occurs at 610 mμ, and the pH range for formation is 4.5–10. The zinc complex also exhibits maximum absorption at 610 mμ, but is only formed above pH 8. Hence, RUSH AND YOE[32] estimated the copper content of the sample on an aliquot of solution at pH 5.2, whilst another estimation at pH 9 gave the combined zinc–copper figure; the zinc figure was obtained by difference.

The present work confirmed the possibility of simultaneously estimating zinc and copper but a slight modification was required. RUSH AND YOE observed that the slopes of the calibration graphs for copper at pH 5.2 and 9.0 were the same, hence the zinc was obtained by direct difference. For this work it was found that the slope of the calibration graph for copper at pH 5.2 was slightly lower than that at pH 9.0. A correction has thus to be made to convert the optical density reading for copper alone at pH 5.2 into an equivalent reading at pH 9.0. If this is done, the zinc figure may be accurately found by difference. The slopes of the calibration graphs for copper at 610 mμ are (1) 0.370 optical density units per μg of copper per ml at pH 5.2 and (2) 0.382 optical density units per μg of copper per ml at pH 9.0.

Cadmium

The residue after elution and fuming down to a few drops was dissolved in 5 ml of 0.01 *M* hydrochloric acid, this solution was transferred to a 100 ml volumetric flask for absorptiometric determination.

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TABLE II

SEPARATION OF LEAD, COPPER, IRON AND ZINC

Ratio Pb:Zn:Cu:Fe	Lead		Zinc		Copper		Iron	
	present µg	found µg	present µg	found µg	present µg	found µg	present µg	found µg
1:1:1:1	500	495	500	494	500	505	500	495
1:1:1:1	400	397	400	405	400	400	400	405
1:1:1:1	200	202.5	200	205	200	203	200	200
	200	198	200	197	200	198	200	203
1:1:1:1	100	97.5	100	100.9	100	101	100	100.5
	100	99.0	100	98.0	100	100	100	101.0
1:1:1:1	50	48.7	50	475	50	45.9	50	49.0
	50	48.9	50	51.3	50	49.0	50	51.8
1:1:1:1	20	19.2	20	19.7	20	187	20	21.5
10:1:1:1	1000	—	100	98.4	100	101	100	99.0
	1000	—	100	101	100	98.2	100	98.5
50:1:1:1	4000	—	80	80.5	80	79.3	80	80.0
	4000	—	80	81.0	80	79.0	80	83.0
100:1:1:1	4000	—	40	41.9	40	37.5	40	41.0
	4000	—	40	43.0	40	37.8	40	38.0

Eriochrome Black T. A procedure exactly analogous to that described for the indirect estimation of lead with Eriochrome Black T was carried out. Beer's law was obeyed over the range 0–5 µg of cadmium/ml, and the slope of the calibration graph was 0.195 optical density units per µg of cadmium per ml at 520 mµ.

Blank chromatograms

The chromatographic solvent removes small quantities of iron and other metals from the filter paper, and it is necessary to apply a blank correction to values obtained for heavy-metal samples[23]. A procedure exactly similar to that employed in ref.[23] was used, and quantitative recovery was always obtained when this correction was applied. The application of an empirical correction factor such as suggested by EBEL[40] is not required.

Analysis of synthetic mixtures

Synthetic binary and ternary heavy-metal mixtures were prepared from solutions of the metal nitrates standardised gravimetrically. The separation of zinc(II), copper(II), iron(III) and bismuth(III) from lead was found to be independent of the amount of lead and the metals may be estimated to within ± 3% for amounts greater than 30 g. Binary mixtures containing lead as the major element together with zinc(II), iron(III), copper(II), or bismuth(III) containing up to 500 parts of lead to 1 part of the "trace metal" have been analysed to within ± 4% for the "trace metal".

TABLE III

SEPARATION OF LEAD, ZINC, COPPER AND BISMUTH

Ratio Pb: Zn: Cu: Bi	Lead		Zinc		Copper		Bismuth	
	present µg	found µg	present µg	found µg	present µg	found µg	present µg	found µg
1:1:1:1	200	203.5	200	207	200	202	200	195
	200	198	200	205	200	198	200	195
1:1:1:1	100	100	100	100	100	101	100	97.5
	100	99	100	103	100	100	100	97.5
	100	100.3	100	102	100	101	100	100
1:1:1:1	50	48.0	50	49.8	50	51.2	50	48.7
	50	50.5	50	53.1	50	48.8	50	48.7
1:1:1:1	20	18.8	20	19.6	20	18.8	20	20.3*
	20	19.8	20	19.2	20	19.7	20	19.4*
10:1:1:1	1000	—	100	97.3	100	98.0	100	99*
	1000	—	100	98.6	100	98.5	100	99*
50:1:1:1	4000	—	80	82.0	80	78.8	80	79.5*
	4000	—	80	81.3	80	79.6	80	78.8*
	2000	—	40	40.8	40	39.4	40	39.3*
	2000	—	40	40.5	40	39.0	40	39.4*
100:1:1:1	4000	—	40	41.3	40	39.0	40	40.2*
	4000	—	40	42.0	40	38.7	40	40.1*

* After acid washing, the paper was eluted with solvent to reduce heavy metal impurities.

TABLE IV

SEPARATION OF LEAD, CADMIUM, COPPER, ZINC, BISMUTH AND MERCURY*

Ratio Pb:Cd:Cu:Zn:Bi: Hg	Lead		Cadmium		Copper		Zinc		Bismuth	
	present µg	found µg	present µg	found µg	present µg	found µg	present µg	found µg	present µg	found µg
1:1:1:1:1:1	200	203.7	200	205	200	203	200	207	200	193
	200	198	200	205	200	201	200	205	200	194
	100	96	100	96	100	99	100	104	100	97
50:1:1:1:1	4000	—	80	75	80	79	80	83	80	78

* Mercury analyses were not carried out.

For separation of more complex mixtures reference should be made to Tables II, III and IV where the results are fully tabulated.

SUMMARY

By using a simple descending-elution technique, in conjunction with an anion exchange separation, it has been found possible to analyse for numerous heavy

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metals (including lead) in lead-containing synthetic mixtures. This method is of particular value for the estimation of small amounts of iron, copper, cadmium, zinc, and bismuth in lead.

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CHROMATOGRAPHY OF PTEROYLGLUTAMIC ACID AND RELATED COMPOUNDS ON ION-EXCHANGE RESINS*

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INTRODUCTION

The high order of biological activity of pteridine compounds makes it desirable to establish the purity of the materials used in biochemical studies. In addition, it would be useful to have a method of purifying milligram quantities of these compounds.

A previous study in this laboratory[1] showed the suitability of ion-exchange chromatography for both analysis and purification of pteroylglutamic acid (folic acid, PGA) and 4-amino-PGA (Aminopterin). Subsequently this work was extended to other conjugated and unconjugated pteridines, including PGA analogs, and a preliminary report was presented[2]. The ion-exchange behavior of these compounds has been compared under similar conditions, and the influence of several variables determined. The most useful procedure appeared to be the adsorption of pteridines from alkaline solution onto Dowex-1-chloride columns, and elution with dilute hydrochloric acid.

There have been several reports of the use of ion-exchange chromatography for pteridines, usually for the preparation of a single compound. SILVERMAN and coworkers[3,4] have used Dowex-1 in the isolation of N^{10}-formyl-PGA and anhydroleucovorin. ZAKRZEWSKI AND NICHOL[5] separated PGA and leucovorin on Dowex-1-acetate. USDIN AND PORATH[6] studied several methods of separating PGA, leucovorin and pteroyltriglutamic acid, including a promising triethylaminoethyl cellulose anion-exchanger. The unconjugated pteridine biopterin was purified on Dowex-50 by PATTERSON, MILSTREY AND STOKSTAD[7].

MATERIALS AND METHODS

Dowex-1, -2, and -50 ion-exchange resins were obtained commercially in the 200–400 size, and in various degrees of cross-linking. Before use, a resin was washed several times by suspending it in water, allowing it to settle, and decanting the water and very fine particles. A slurry of the washed resin was poured into a glass tube containing a sintered glass disc covered by a disc of coarse filter paper.

Dowex-1 or -2 columns in the chloride form were prepared by washing with at

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least 200 ml of N HCl, followed by water until the washings were nearly neutral. The washing with acid and water was repeated after each column separation. In some cases new columns were washed with N NaOH and water prior to the acid wash, but this step was not essential. After washing was completed, excess resin was removed to give a column of the desired length, and a loose plug of glass wool was placed slightly above the resin surface.

Dowex-1-formate was prepared by washing the chloride resin with 2 M sodium formate until the effluent gave a negative chloride test, then with 4 N formic acid until the optical density of the effluent fell to less than 0.01. A final water wash removed excess formic acid. Columns were washed with 4 N formic acid and water after each separation run. It is important to avoid rubber connections in the system when formic acid solutions are used. Short lengths of Tygon plastic tubing, previously washed with 4 N formic acid, may be used.

Dowex-1-hydroxide was prepared by washing the chloride resin with several hundred ml of N NaOH, followed by water. Dowex-50-hydrogen form was prepared by washing with 4 N HCl and water.

Columns 1 cm in diameter and 5 to 14 cm in length have been used in these studies. Although these columns will adsorb large amounts of pteridines (a 7 cm long column of Dowex-1 adsorbs more than 100 mg of PGA from solution), the usable chromatographic capacity is very low. For columns of this cross-sectional area (0.8 cm^2), it appears that less than 5 mg of these compounds must be used in order to obtain complete separation of peaks. Larger amounts of material may be chromatographed on columns of correspondingly larger cross-sectional area.

The interstitial volume (holdback volume) was determined by a method similar to that of RIEMAN AND LINDENBAUM[8]. A column was washed thoroughly with 0.5 N HCl, and all acid above the resin removed. Washing with water to remove the acid, and titration of this wash, allowed calculation of the ml of acid in the column. Dowex-1-chloride of 4% cross-linking was found to have an interstitial volume of 51% of the total volume of the resin bed in a column.

Compounds to be chromatographed were suspended in a few ml of water, and dissolved by the addition of ammonium hydroxide to pH 8 to 9. The solution was allowed to run into the column and was washed in with a few ml of water. Eluants were added by gravity from bottles above the column, to give flow rates of 0.3–0.7 ml/min (0.4–0.9 ml/min/cm^2). The column and fraction collector were covered to exclude light. Fractions were collected on a time basis rather than with a photoelectric drop counter, to avoid exposure to light. The optical density of each fraction was determined in the Beckman DU spectrophotometer, usually at 300 mμ. Fractions to be studied further were usually neutralized; these solutions are generally of sufficient concentration to be used directly for biological studies. Fractions to be chromatographed on paper were evaporated to dryness in a vacuum desiccator at room temperature in the dark. Paper chromatography was carried out on Whatman No. 1 paper, in 0.1 M aqueous phosphate buffer of pH 7.0, and spots were located under ultraviolet light.

RESULTS

Choice of resin

In preliminary tests, it was found that PGA was adsorbed from solution at pH 5 on a column of Dowex-50-hydrogen form cation-exchanger. Some of the compound was eluted with 3 *N* hydrochloric acid, but recovery was poor. Cation-exchangers were not studied further, because of the insolubility and instability of most of these compounds in the required concentrations of acid. In a single test with Dowex-2-chloride, PGA was satisfactorily adsorbed and eluted under the same conditons used with Dowex-1.

The remainder of the studies described here were carried out with Dowex-1. The percentage of cross-linking of the resin has a considerable effect on the chromatograms obtained with conjugated pteridines. A 10% cross-linked Dowex-1 (Dowex-1-X10) did not give as sharp elution peaks as 4% resin (Dowex-1-X4) under the same conditions (Fig. 1). A further difficulty experienced with 10% cross-linked resin was the incomplete elution of material in the peak fractions, with the remainder appearing in the *N* hydrochloric acid wash at the conclusion of the run. This was particularly true of experiments done at lower temperatures, or where the room temperature dropped considerably during a run. Although Dowex-1 of from 2 to 10% cross-linking has been used successfully, resin with about 4% cross-linking appears to give the most consistent results.

Temperature

The effects of temperature were studied by surrounding the ion-exchange column with a jacket through which water from a constant temperature bath was circulated. Fig. 2 illustrates the sharpening of the peak obtained when PGA is eluted at 35° (curve B) as compared with 25° (curve C), using similar flow rates. Increasing the

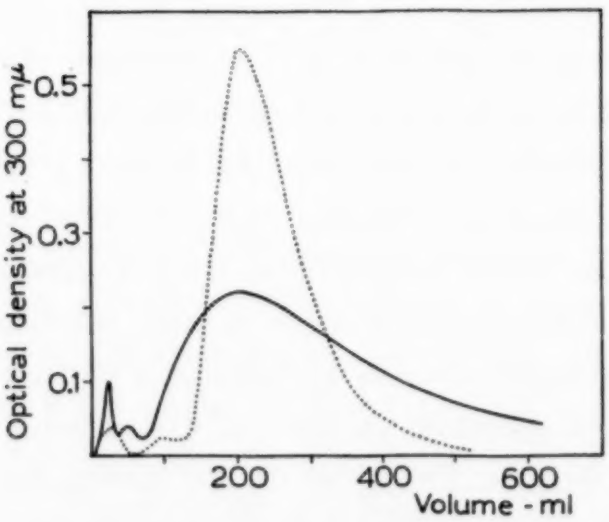

Fig. 1. Effect of resin cross-linking on elution of 2 mg PGA with 0.05 *N* HCl at 35°. 8 × 1 cm Dowex-1-chloride. —— 10% cross-linked, flow rate 0.35 ml/min; ····· 4% cross-linked, 0.45 ml/min.

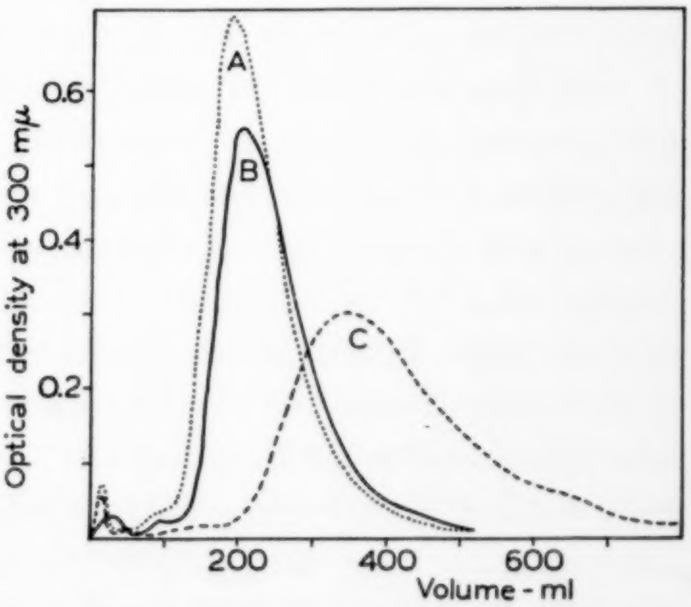

Fig. 2. Effect of temperature on elution of 2 mg PGA from 8 × 1 cm Dowex-1-X4-chloride with 0.05 *N* HCl. A: 35°, flow rate 0.73 ml/min. B: 35°, 0.45 ml/min. C: 25°, 0.53 ml/min.

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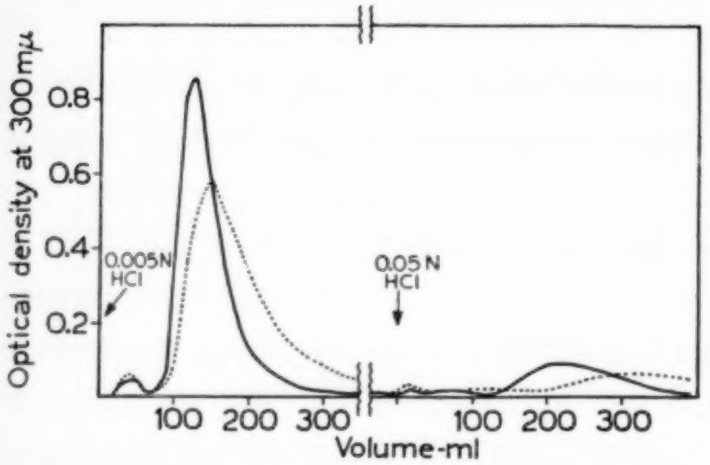

Fig. 3. Effect of temperature on elution of 2 mg Aminopterin from 8 × 1 cm Dowex-1-X4-chloride, 0.60 ml/min. —— 35°, ······ 25°.

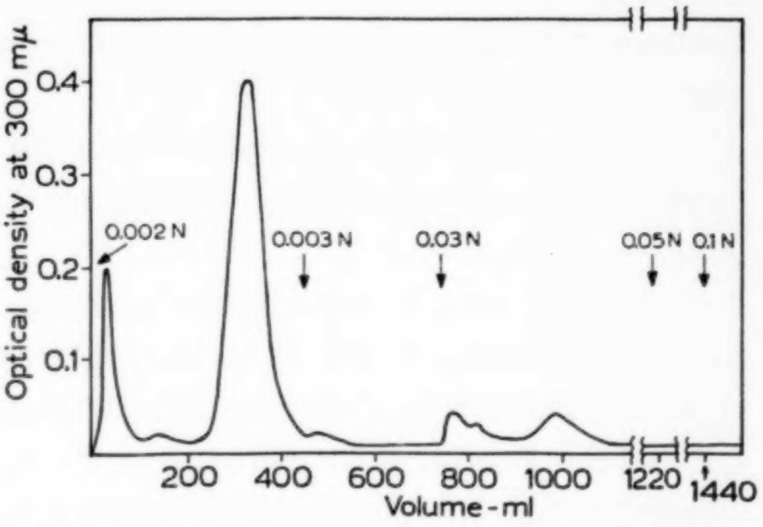

Fig. 4. Amethopterin, 1.5 mg, 7 × 0.9 cm Dowex-1-X2-chloride. Eluted with HCl, 0.50 ml/min.

flow rate at the higher temperature (curve A) results in a further slight narrowing of the peak.

The elution patterns of compounds which are eluted with more dilute acid are less markedly affected by temperature, as shown by the elution curves for Aminopterin (Fig. 3). The broad peak which is eluted with 0.05 *N* hydrochloric acid is PGA, present as an impurity. Most of the experiments described in this paper were done without temperature control, at room temperatures of approximately 25°.

Elution from Dowex-1-chloride with hydrochloric acid

The free pteridines tested were found to be eluted more readily than the conjugated pteridines. Thus xanthopterin, leucopterin, and biopterin were adsorbed from alkaline solution on Dowex-1-chloride, were not washed off with water, but were brought off with small volumes of 0.001 *N* hydrochloric acid. Among the PGA analogs, those in which an amino group replaces the 4-hydroxyl are more readily eluted. 4-Amino-N^{10}-methyl-PGA (Amethopterin) is eluted with 0.002 *N* hydrochloric acid (Fig. 4), and Aminopterin with 0.005 *N* hydrochloric acid (Fig. 3), while elution of PGA requires 0.05 *N* acid. The small peak preceding Aminopterin in Fig. 3 is a minor unidentified impurity. The fact that this material and PGA have been found in even the best samples of Aminopterin examined may reflect the instability of this analog on storage. The ultraviolet absorption spectra in acid and alkali found for Aminopterin purified on a Dowex-1-chloride column appear to be identical with those presented by SEEGER *et al.*[9].

Methopterin (N^{10}-methyl-PGA) is eluted with 0.03 *N* hydrochloric acid. Both PGA and 5-formyl-5,6,7,8-tetrahydro-PGA (Citrovorum factor, leucovorin) are eluted with 0.05 *N* hydrochloric acid, and it has not been possible to separate a mixture of these two compounds by hydrochloric acid elution. Since the acid lability of leucovorin[10] results in rapid decomposition even in 0.05 *N* acid (as shown by changes in ultraviolet absorption, and paper chromatograms), each fraction containing this compound would have to be neutralized immediately. A more practical eluant is sodium chloride, which avoids the use of acid, and separates PGA and leucovorin, as described below.

N^{10}-Formyl-PGA is eluted more rapidly than PGA with 0.05 *N* hydrochloric acid, and the two compounds may be separated completely by use of this solvent (Fig. 5).

Pteroic acid is very firmly adsorbed on Dowex-1, and only a fraction of it is removed with 0.5 *N* or 1 *N* hydrochloric acid. Complete elution from the resin requires 1 *N* hydrochloric acid at elevated temperatures, and this is not considered a practical procedure. Pteroic acids which may be present as impurities in the other compounds studied would therefore not contaminate the products purified by the above procedures involving acids of less than 0.1 *N*.

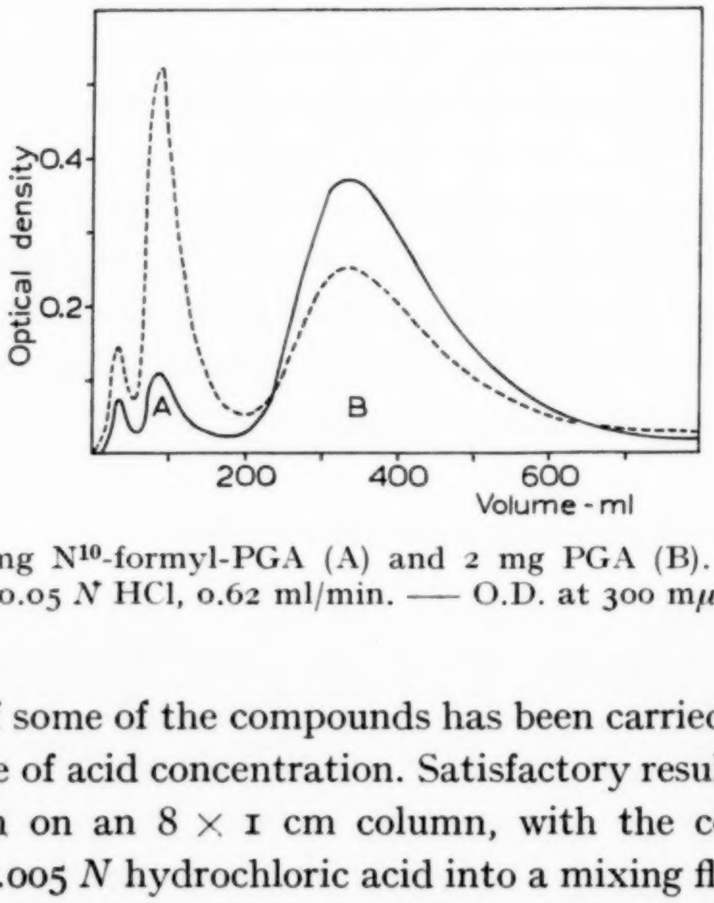

Fig. 5. Separation of 1 mg N^{10}-formyl-PGA (A) and 2 mg PGA (B). 9 × 1 cm Dowex-1-X4-chloride. Eluted with 0.05 *N* HCl, 0.62 ml/min. —— O.D. at 300 mμ, ------ O.D. at 250 mμ.

Gradient elution of some of the compounds has been carried out as an alternative to the step-wise change of acid concentration. Satisfactory results were obtained with 2 mg of Amethopterin on an 8 × 1 cm column, with the concentration gradient provided by running 0.005 *N* hydrochloric acid into a mixing flask containing 200 ml of water, and then to the column. Under similar conditions, PGA was eluted when 0.1 *N* hydrochloric acid was run into the mixing flask of water. In general, gradient elution appeared to be less sensitive in detecting trace impurities, and did not offer any particular advantage to offset the requirement for a more complicated experimental arrangement.

Elution from Dowex-1-chloride with hydrochloric acid and ethanol

The principal disadvantage of hydrochloric acid eluants is the low solubility of the pteridines in acid. When it is necessary to increase the capacity of the chromatographic system, alcohol may be added to the eluants. In the case of PGA, the presence of alcohol reduces the volume of solution required to elute the compound, and sharpens the peaks. With 5, 10, and 20% ethanol in 0.05 *N* hydrochloric acid, the peak fraction of PGA occurred at 240, 180, and 80 ml, respectively. When 20% ethanol was used, it was possible to reduce the acid concentration by one-half, without impairing resolution

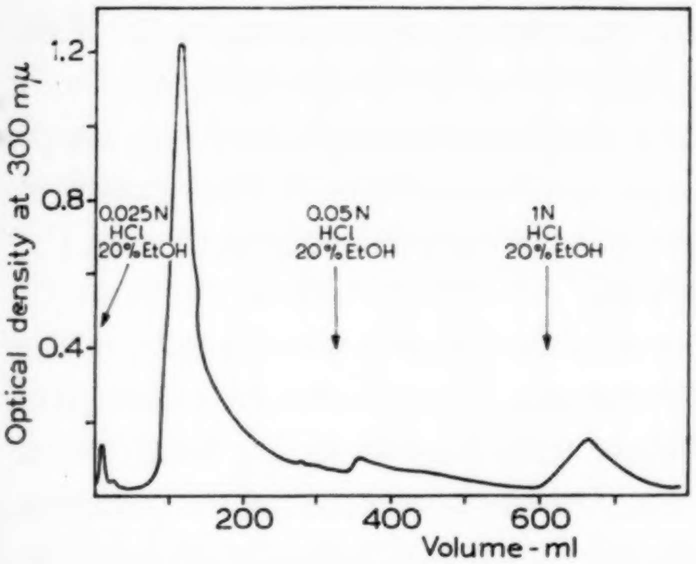

Fig. 6. Elution of 3 mg PGA with HCl–ethanol. 7 × 0.9 cm Dowex-1-X7.5-chloride, 0.12 ml/min.

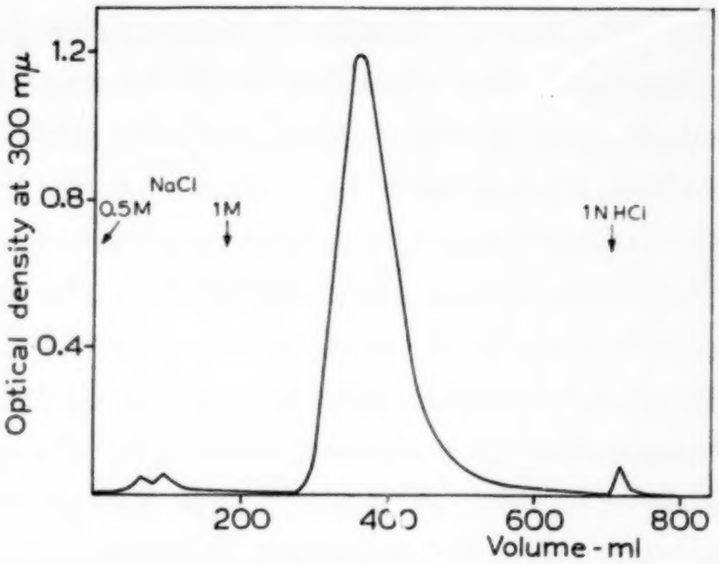

Fig. 7. Elution of 3 mg PGA with NaCl. 7 × 0.9 cm Dowex-1-X7.5-chloride, 0.27 ml/min.

or recovery of PGA (Fig. 6). When Aminopterin was chromatographed with 20% ethanol added to the 0.005 *N* hydrochloric acid usually used for elution, the compound was removed too rapidly for good resolution. Eluting with 10% ethanol in 0.005 *N* acid was more satisfactory. These solvents were not studied further.

Elution from Dowex-1-chloride with sodium chloride

The removal of pteridine compounds from anion-exchangers with acid requires solutions of pH 1.5 to 3. In some cases, notably leucovorin, this pH is low enough to cause chemical changes, and make it difficult to recover the separated compounds. An alternative to desorbing the pteridines by lowering the pH is the use of solutions of higher ionic strength. Thus it is possible to elute leucovorin with 0.5 *M* sodium chloride with no acid added, while PGA is eluted with larger volumes of 0.5 *M* or smaller volumes of 1 *M* sodium chloride (Fig. 7). A mixture of these two compounds is separated completely by use of these eluants. Aminopterin is eluted with larger volumes of 1 *M* sodium chloride than are required for PGA elution.

Elution from Dowex-1-formate with formic acid

It has been found that the formic acid solvents will separate the monoamino- from the diamino-folic acid compounds, but there is no resolution within each group. Aminopterin and Amethopterin were eluted with 0.05 *N* formic acid. When a mixture of these two compounds was chromatographed on a column of Dowex-1-X4-formate 8 cm in length, both compounds appeared in a single peak. Paper chromatography showed the first fractions of the peak to contain more Amethopterin than Aminopterin, and the last fractions to have the opposite proportion, but there was no separation. In a similar manner Methopterin and PGA are eluted with 1.5 *N* formic acid, but are not separated when a mixture of the two is chromatographed.

During the course of the work with formic acid solvents, it was noticed that fractions containing PGA, when evaporated and chromatographed on paper, showed the presence of another compound with light blue fluorescence at R_F 0.81. This compound was identified as N^{10}-formyl-PGA by its R_F, its spectrum in acid and alkali, and by the change in spectrum to that of PGA upon standing 24 h in 0.1 *N* sodium hydroxide[11]. It is not surprising that there was no evidence of formylation of the analogs, since Aminopterin is eluted with much more dilute formic acid, and the other compounds are methylated in the 10-position.

In a series of test tube experiments, it was found that approximately 23% of the PGA was formylated after standing 24 h at room temperature in 2 *N* formic acid, and evaporation in a vacuum desiccator. In 4 *N* formic acid there was 44% formylation after 2 h, and 48% after 24 h. The presence of Dowex-1-formate in the reaction mixture appeared to inhibit the formylation to some extent, and there was no formylation by the resin itself without the presence of formic acid.

DISCUSSION

The adsorption of pteridine compounds on the chloride form of an anion-exchanger, and elution with dilute hydrochloric acid, has proved to be the most satisfactory of the methods tested in this laboratory. When used with the proper regard for the low capacity of the solvents, and the influence of temperature, this procedure gives highly reproducible results. It is a sensitive method for detecting impurities, readily showing the presence of a component which contains 0.1% of the total optical absorption of the starting material, if the impurity is separated from other peaks. The use of sodium chloride (also described by SILVERMAN *et al.*[3]) as eluant for compounds sensitive to acid is similar to the method of ZAKRZEWSKI AND NICHOL[5], using acetate buffers, and provides an alternative solvent system.

The recovery of known compounds adsorbed on Dowex-1-chloride columns has ranged from 60 to 100%, depending on experimental conditions. Low temperature appears to be the principal reason for incomplete elution, and recovery was essentially complete in experiments done at controlled temperatures. The presence of pteroic acid as an impurity would also contribute, since little or none of it is eluted from the resin.

Most of the compounds separated on the columns may be recovered unchanged in the effluent. It was shown previously[1] that Aminopterin is not deaminated to PGA during chromatography on Dowex-1-chloride, since purified Aminopterin may be rechromatographed without showing the presence of PGA. In the present study PGA, too, has been found to be stable to chromatography in this system. As noted above, leucovorin is rapidly destroyed by an acid solvent, but may be chromatographed with sodium chloride if the high salt concentration is not objectionable. The formylation of PGA when it is chromatographed in the formic acid system is an obvious objection to this procedure. In view of the biological activity of formylated pteridines, formate chromatography should be used with caution.

A number of solvents in addition to those discussed were tested briefly, and found to be unsatisfactory. Aminopterin was not eluted from Dowex-1-X2-chloride with 0.2 M ammonium hydroxide–0.025 M ammonium chloride buffer of pH 11.3. PGA was not eluted from the hydroxide form of Dowex-1 with 1 N ammonium hydroxide, nor from the chloride resin with 1 M ammonium carbonate or 0.5 M sodium citrate.

It has not proved possible to use this chromatographic method directly on crude extracts of natural materials. The presence of nucleotides, amino acids, and other compounds which are adsorbed and eluted under these same conditions makes impossible the detection of the much smaller amounts of pteridines usually present. The method should be applicable to material which has gone through some preliminary purification, such as charcoal adsorptions. The sensitivity and specificity of the method could be increased by the use of a biological assay of effluent fractions, in addition to optical density measurements.

ACKNOWLEDGEMENTS

The authors are grateful to the Lederle Laboratories Division, American Cyanamid Company, for generous supplies of the pteridine compounds used in this study. The assistance of MARJORIE NELSON, CATHERINE CONNELLY, and MARTHA ROBERTS in various phases of this work is gratefully acknowledged.

SUMMARY

Techniques are described for analysis and purification of free and conjugated pteridines by anion-exchange chromatography. The following compounds are adsorbed on Dowex-1-chloride and eluted with very dilute HCl or with NaCl: biopterin, leucopterin, xanthopterin, PGA, leucovorin, N^{10}-formyl-PGA, Aminopterin, Amethopterin, Methopterin. During chromatography in the formic acid system, appreciable amounts of PGA are formylated to N^{10}-formyl-PGA.

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PAPER CHROMATOGRAPHY OF STEROIDS IN SYSTEMS WITH ETHYLENE GLYCOL AS THE STATIONARY PHASE

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In paper chromatography of corticosteroids and 17-ketosteroids, the systems with more polar organic solvents, such as propylene glycol, formamide, dimethylformamide, phenylcellosolve, cellosolve, 1,3-butanediol, etc., in the stationary phase are routinely used.

Ethylene glycol in the stationary phase (ethylene dichloride as mobile phase) has been used as early as 1951 by BOSCOTT[1] for paper-chromatographic separation of urinary phenols and œstrogens. The chloroform/ethylene glycol system has been described for paper chromatography of cardenolides[2]. Ethylene glycol in the stationary phase for the separation of corticosteroids was first proposed for partition chromatography on columns[3, 4], while recently it has been used for effective separations, especially of aldosterone, by paper chromatography, toluene being the mobile phase[5].

Systems with ethylene glycol have also been successfully used for the separation of 17-ketosteroids[6], œstrogens and corticosteroids[7]; their use will, therefore, be dealt with in some detail.

EXPERIMENTAL

Steroids in chloroform solution were applied to sheets of Whatman No. 1 paper (15 × 48 cm). After the application of the solution the strips were soaked in a 30% solution of ethylene glycol in methanol, blotted between filter paper and exposed to air at room temperature for 20 minutes. The chromatograms were then transferred to the chromatography tanks, saturated with vapours of the mobile phase, and developed by the descending technique at 17–20°.

For chromatographic separation of corticosteroids, toluene or carbon tetrachloride saturated with ethylene glycol, and for 17-ketosteroids petroleum ether (b.p. 45–65°) were used.

The steroids were detected in the usual ways, corticosteroids by U.V. photocopying or by treatment with a solution of blue tetrazolium, 17-ketosteroids with *m*-dinitrobenzene reagent.

RESULTS AND DISCUSSION

Ethylene glycol is only slightly soluble in carbon tetrachloride and toluene, and in comparison with propylene glycol only sparingly miscible with chloroform; consequently systems with ethylene glycol as the stationary phase may even be used for the separation of very polar steroids[1, 2, 5]. On the other hand it may be possible, by using an appropriate mobile phase, *e.g.* hexane or petroleum ether, to separate even less polar C_{19}-steroids.

The R_F values of some corticosteroids in the carbon tetrachloride/ethylene glycol and the toluene/ethylene glycol systems are shown in Table I, and those of some 17-ketosteroids in the petroleum ether/ethylene glycol system in Table II. In these determinations the sheets were always impregnated with a 30% solution of ethylene glycol in methanol. Impregnation with this solution gives the highest rate of flow of the mobile phase with the most sharp separation of steroids that are frequently

TABLE I

R_F VALUES AND MOBILITIES OF SOME CORTICOSTEROIDS AND ŒSTROGENS IN ETHYLENE GLYCOL SYSTEMS

Steroid	*Toluene/ethylene glycol*		*Carbon tetrachloride/ethylene glycol*	
	R_F	*Mobility (cm/20 h)*	R_F	*Mobility (cm/20 h)*
Tetrahydrocortisol		4.5		0.1
Tetrahydrocortisone		8.2		0.5
Cortisol	0.03	13.2	0.01	1.6
Aldosterone	0.07	18.9	0.08	19.0
Cortisone	0.10	25.3	0.10	26.2
11-Desoxycortisone	0.43		0.15	
Corticosterone	0.51		0.20	
11-Desoxycorticosterone	0.88		0.88	
Cortisone acetate	0.70		0.26	
11-Desoxycortisone acetate	0.85		0.40	
11-Desoxycorticosterone acetate	0.96		0.98	
Œstrone			0.50	
Œstradiol			0.12	

TABLE II

R_F VALUES OF KETOSTEROIDS IN A PETROLEUM ETHER/ETHYLENE GLYCOL SYSTEM

Steroid	R_F
Androstane-3α,11β-diol-17-one	0.06
Ætiocholan-3α-ol-11,17-dione	0.13
Δ^4-Androsten-11β-ol-3,17-dione	0.20
Δ^4-Androstene-3,11,17-trione	0.24
Δ^5-Androsten-3β-ol-17-one	0.47
Androstan-3β-ol-17-one	0.56
Ætiocholan-3α-ol-17-one	0.54
Androstan-3α-ol-17-one	0.67
3,5-Cycloandrostan-6β-ol-17-one	0.76
Androstane-3,17-dione	0.85
3β-Chloro-Δ^5-androsten-17-one	0.94
Testosterone	0.22
17-Methyltestosterone	0.43
Pregnane-3,20-dione	0.92
Progesterone	0.84

estimated. The influence of concentration of the impregnating solution on the R_F values of some 17-ketosteroids is apparent in Fig. 1.

The influence of the saturation rate of the tank and variations of temperature on the rate of flow of the mobile phase is especially marked when very volatile solvents, e.g. petroleum ether, are used. In chromatography of 17-ketosteroids the optimal rate of flow is 30–35 cm in 2½ hours. Within this velocity range the R_F values are readily reproducible. In solvent systems with carbon tetrachloride or toluene the liquid front reaches 35 cm in 3–4 hours. For the separation of steroids the use of ethylene glycol has the advantage that a high velocity of partiti[illegible] is obtained.

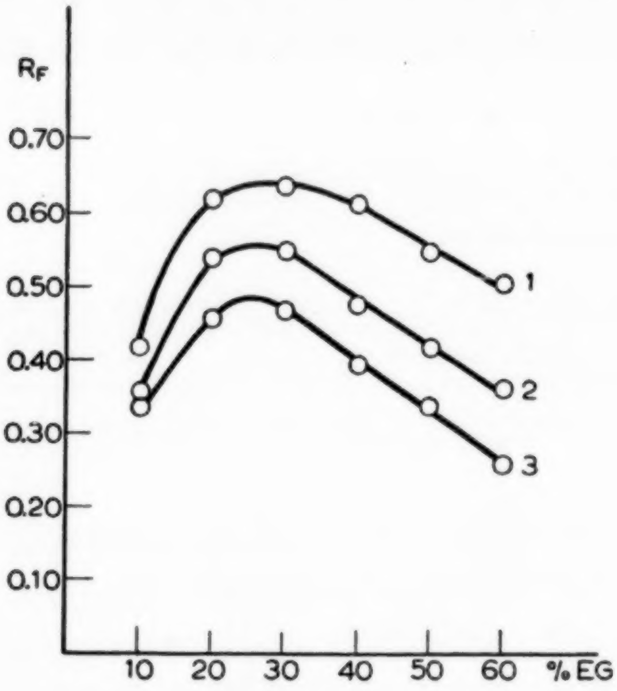

Fig. 1. Effect of the ethylene glycol (EG) concentration on the R_F values of some 17-ketosteroids. 1 = androsterone; 2 = epiandrosterone; 3 = dehydroepiandrosterone. Mobile phase: petroleum ether.

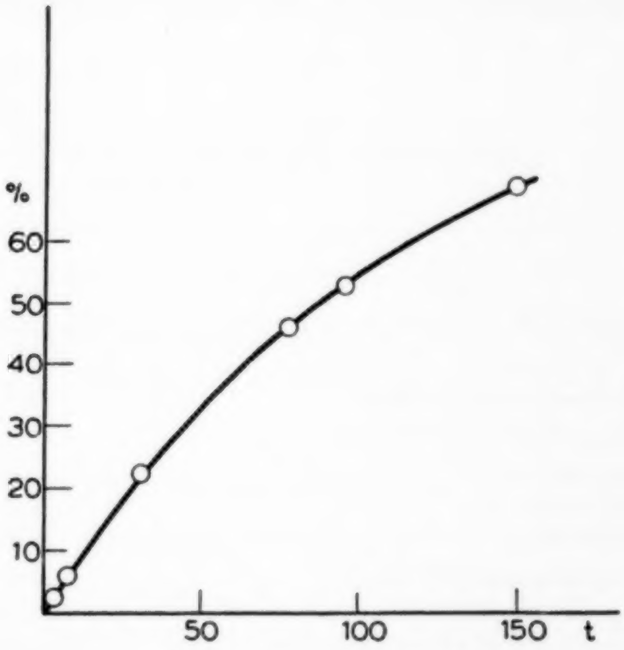

Fig. 2. Effect of increase of time of evaporation on ethylene glycol loss from the impregnated paper. Time t in hours. % = percentage of ethylene glycol lost.

The capacity of Whatman No. 1 paper, on which the separated steroids appear as round or oval spots, depends to a certain extent on the nature of the investigated substance, on the amount of ethylene glycol in the stationary phase, and on the

TABLE III

CAPACITY OF WHATMAN NO. 1 PAPER FOR SOME STEROIDS. EFFECT OF ETHYLENE GLYCOL CONCENTRATION IN THE IMPREGNATING SOLUTION

Results are expressed as μg steroid/spot

Steroid	*Conc. of ethylene glycol*			
	10%	*30%*	*50%*	*70%*
Cortisol*	50	100	100	150
Cortisone*	40	350	380	420
Δ^5-Androsten-3β-ol-17-one**	150	350	400	450

* Mobile phase: toluene.
** Mobile phase: petroleum ether.

mobile phase. Values obtained for some steroids are shown in Table III. The capacity of Whatman No. 3 paper exceeds by 50% the capacity of Whatman No. 1 paper.

In the case of identically impregnated sheets, the amount of ethylene glycol in the stationary phase depends on the concentration of the solution of ethylene glycol in methanol. During the impregnation with a 10% solution the stationary phase of ethylene glycol amounts to about 7% of the weight of the paper, with a 30% solution it amounts to 28% and with 50% solution to 45%.

Ethylene glycol evaporates from the paper strips more quickly than formamide. This facilitates the removal of the impregnating agent from the sheets. However, during the development of the chromatogram the evaporation from the sheet is never so marked that the effect on the R_F values of the loss of ethylene glycol is significant. The evaporation of ethylene glycol from the paper strips is illustrated in Fig. 2, which shows the influence of time on ethylene glycol loss at room temperature in a closed chromatography tank.

Ethylene glycol systems have been successfully used not only for the separation of pure steroids, but also for the separation of corticosteroids and 17-ketosteroids from biological material, especially extracts from urine.

Ethylene glycol is available in a grade of satisfactory quality and it does not influence the majority of color reactions used for the quantitative colorimetric determination of steroids in urine.

SUMMARY

The use of ethylene glycol as the stationary phase for the separation of corticosteroids and of 17-ketosteroids by paper chromatography is proposed. Some characteristics of systems with ethylene glycol in the stationary phase are described.

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IONOPHORETIC PATTERN IN TWO-DIMENSIONAL ELECTROPHORESIS

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As two-dimensional electrophoresis requires uniformity of all forces acting on migration over the entire surface of the curtain, it is important to maintain homogeneous ionic distribution over the whole supporting medium. The first requirement is a correct hydrodynamic flow. This presupposes the correct rinsing of the electrodes and a correct central buffer-feeding, such as is realized with cascade electrodes[1,2] and trickle-feeding[3] (Fig. 1). The cascade electrodes as depicted in Fig. 2 have a hydrostatic pressure that is identical over their entire height and are in equilibrium with the central buffer flow of the curtain. The buffer circulation removes the electrolysis products of the electrodes efficiently. Moreover, the buffer of the cathode system (A) can be separated from the anode system (C) and from the central buffer system (B). This allows the use of different buffers in compartments A, B and C in one experiment.

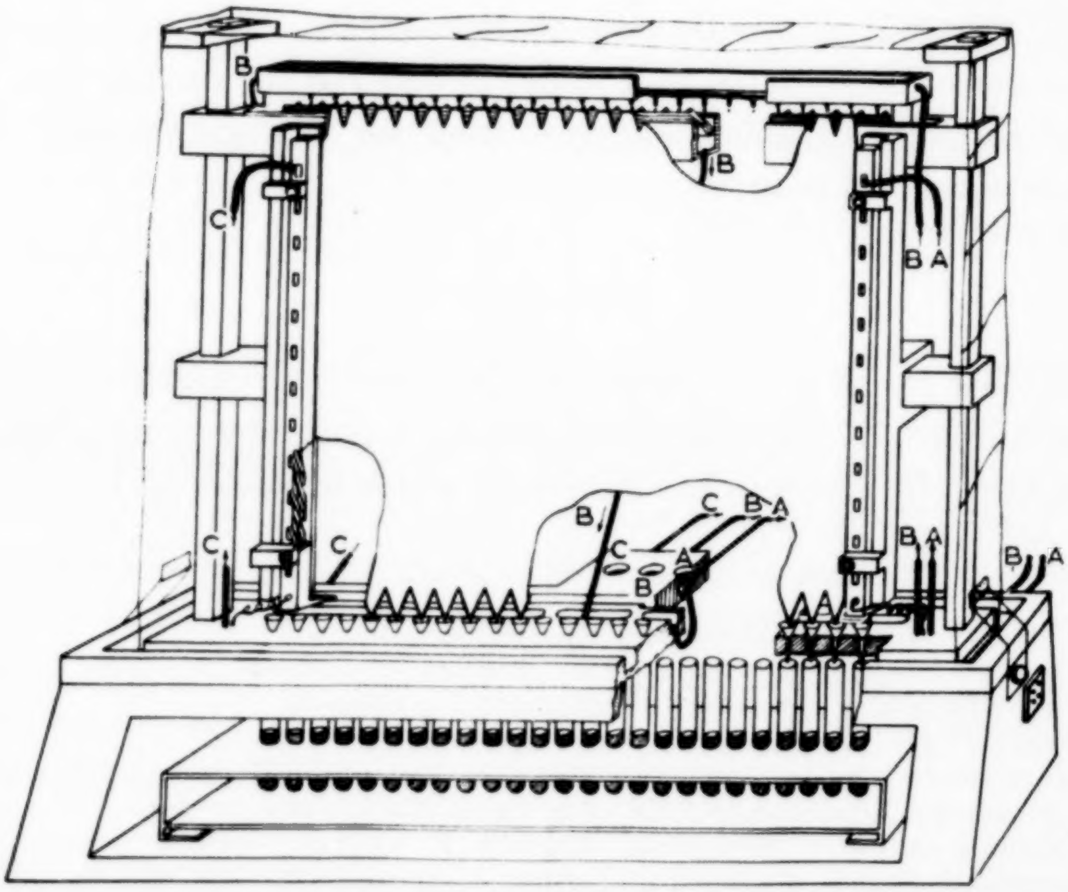


Fig. 1. Two-dimensional electrophoresis apparatus. Electrophoretic chamber with cascade electrodes and trickle-feeding of the curtain. One buffer circuit (A) feeds one electrode, a second (B) the middle field and a third (C) the other electrode.

Trickle-feeding of the curtain eliminates the electrical shunt, which otherwise exists between the electrodes if there is a buffer trough (Fig. 3).

References p. 319.

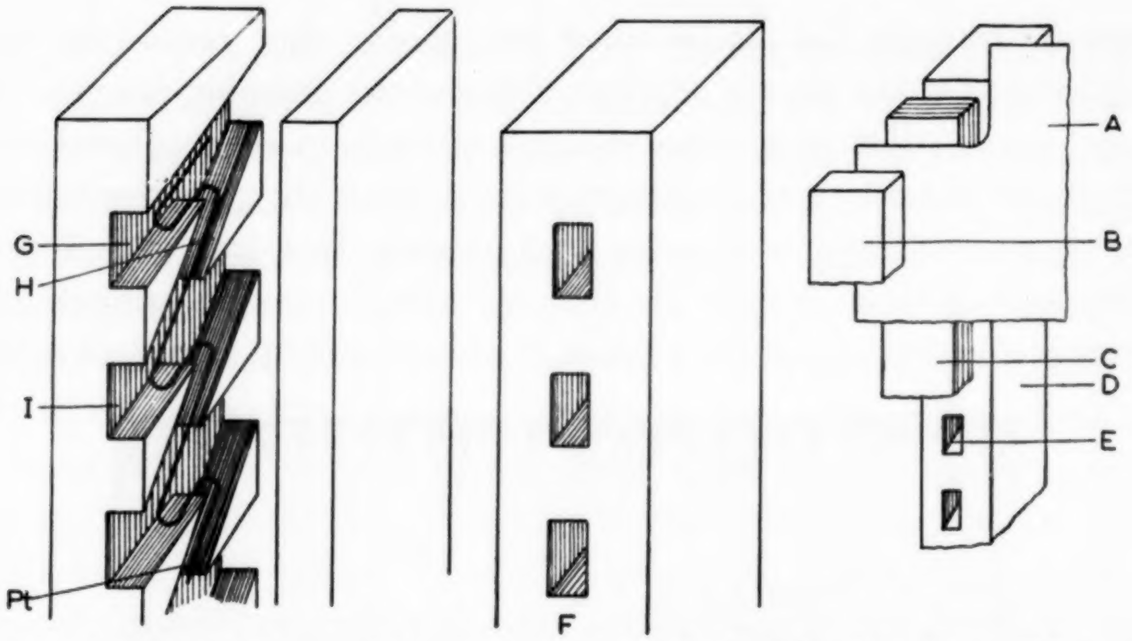


Fig. 2. Cascade electrodes. Schematic view from the back. On the right the position of the paper can be seen. The paper A is clamped between the plate B and the electrode carrier D. The contact with the buffer occurs at the windows E. Some paper strips C ensure contact between the electrode carrier and the paper. In the middle the electrode carrier (F) is shown, the structure of which can be seen in the vertical section on the left. The buffer led into the first groove (G) feeds the paper through the window, and after filling this groove runs through the overflow (H) into the second groove (I) and so on. Thus the electrode wire (Pt) is efficiently rinsed.

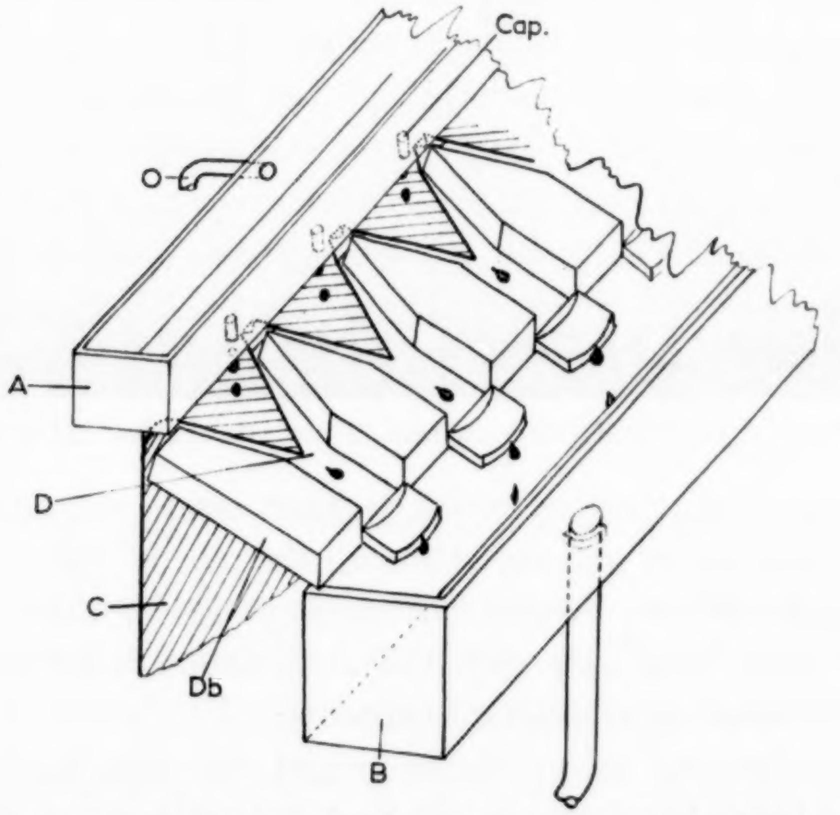


Fig. 3. Trickle-feeding of the curtain. Buffer from the trough A, equipped with the overflow O, trickles through the capillaries (Cap) onto the teeth cut in the upper edge of the curtain C. Each tooth of the curtain lies in a separate channel D of the draining board (Db). The liquid from all the channels is collected in the gutter B.

The electrophoretic chamber is isolated from the surrounding air by means of a cover made of a thin plastic sheet. A water-seal underneath this cover ensures that it is hermetically closed. The buffers are withdrawn by means of a siphon, thus avoiding loss of vapour-saturated air from the chamber.

The application of spots of sample or the measurement of potentials can be accomplished at any place on the curtain without affecting vapour saturation by simply pricking through the plastic cover and closing the needle hole with scotch tape. The pliability of the plastic prevents a rise of the chamber pressure during the warming-up period, which is a common cause of leaks in electrophoretic chambers.

Small plastic funnels with capillary ends passing through the bottom of the chamber allow the collection of samples from outside. As a drop of buffer remains in the capillary ends of these funnels the chamber always remains hermetically closed.

The continuous feeding of the sample is carried out by means of a filter paper wick dipping into a small cuvette, which is fixed inside the chamber and can be filled at the right moment by puncture through the plastic wall.

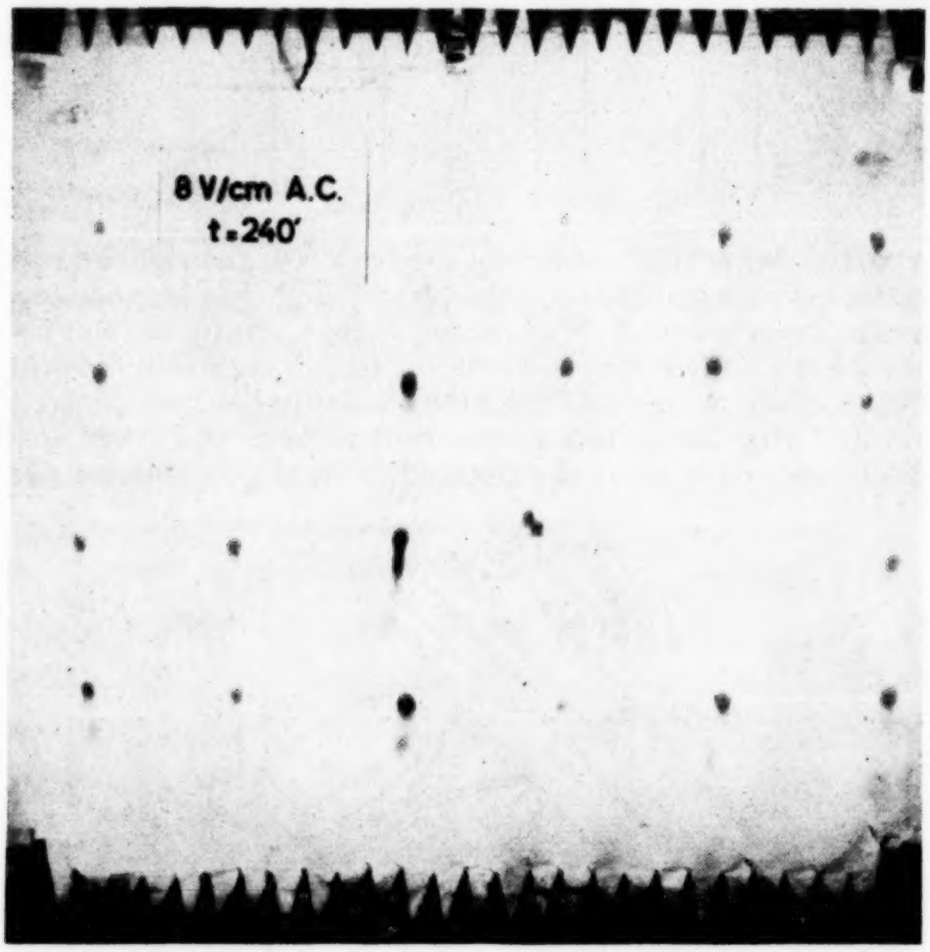


Fig. 4. Eosin spots on a curtain under alternating current.

The power supply delivers a direct current up to 900 V, which shows fluctuations of less than $1^0/_{00}$ when examined with the oscilloscope. This current is stabilized either at constant voltage or at constant intensity.

With this experimental set-up we examined the influence of *evaporation* and *ionophoresis* successively. Evaporation will be discussed first.

In an ideal apparatus evaporation should be identical at any point of the supporting medium. Evaporation can be considered (a) in the horizontal direction, *i.e.* from the electrodes towards the center and (b) in the vertical direction from top to bottom of the curtain. Evaporation in the horizontal direction was examined by chromatographing eosin spots, using the cascade electrodes and applying alternating current. It was found that there is no evaporation if the equilibration period is sufficiently long (*i.e.* about six hours) (Fig. 4). In the vertical direction there is evaporation towards the bottom, as is shown in the same figure by the shorter length of the chromatographic spots the nearer they are to the bottom of the paper.

During electrophoresis, by which we mean the migration of macromolecules, the migration of the buffer ions, called ionophoresis, must be considered. Ionophoresis is often the cause of serious technical difficulties. The pH and ionic strength of the buffer as well as the electrical conditions that were established at the beginning of the experiment, are often disturbed by a shift of the buffer ions, which modifies the ionic distribution of the original buffer. In fact, many attempts to apply either high voltage or a new type of buffer are defeated by ionic movements, followed by salt-deposits inside the supporting medium, resulting in high conductivity and still worse working conditions. This may even lead to burning of a paper medium or to the breaking up of gelose.

There are two main causes of changes in the pH: electrolysis and ionophoresis. Changes due to electrolysis can be completely avoided if cascade electrodes are used. With these electrodes continuous and perfect washing of the electrode wire is combined with perfect hydrodynamic conditions: there is no flooding of the curtain even if the electrodes are very tall.

Thus the only remaining problem is the modification of the original ionic pattern of the buffer through ionophoresis[4,5]. Just as evaporation in the vertical direction of the curtain is an unavoidable difficulty, ionophoresis is an unavoidable phenomenon, which we must try to understand, because it explains important changes in conductivity, ionic strength and pH, which ultimately may ruin an experiment.

A previous study on the distribution of the sodium ion during zone electrophoresis and two-dimensional or collecting electrophoresis[6,7], led us to consider the behaviour of both anion and cation, as well as the conductivity of the supporting medium over the entire surface of large two-dimensional fields. It should be noted that these experiments were not intended for exact calculations of equivalency of the constituent ions. They do, however, make it possible to determine where the ions accumulate as a result of ionophoresis.

I. INVESTIGATION OF IONOPHORESIS WITH A SINGLE BUFFER

In a first series of experiments the paper was cut up into fragments according to a given plan and then *sodium* was determined after elution (Fig. 5)[7]. In a subsequent experiment *sodium* was determined by flamephotometry, *veronal* by its adsorption in the

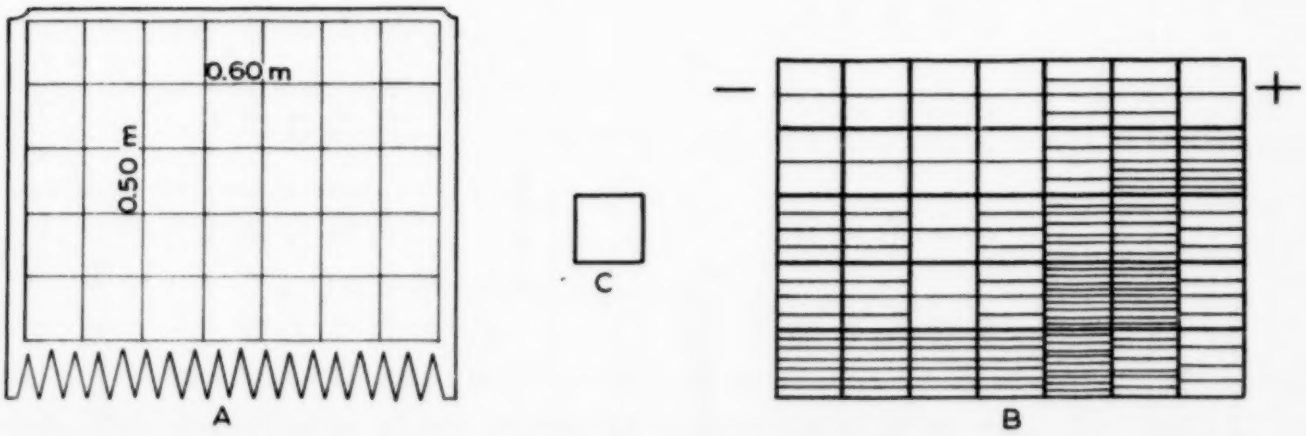


Fig. 5. Sodium distribution in two-dimensional electrophoresis. After the run the curtain is cut into rectangles of 8 × 8.5 cm, according to A. In B sodium contents higher than that of the original buffer C are indicated. The rectangles are shaded according to the sodium content.

References p. 319.

TABLE I

IONIC PATTERN AND CONDUCTIVITY IN TWO-DIMENSIONAL ELECTROPHORESIS

With Na-veronalate/veronal buffer																				
*Sodium**							*Veronal***							*Conductivity****						
—						+	—						+	—						+
61	61	52	53	52	67	61	46	16	44	38	36	66	64	17	15	18	18	16	19	20
44	48	48	42	55	60	42	40	34	36	40	50	66	80	13	14	15	13	15	15	14
41	48	38	39	42	52	65	38	46	42	40	50	54	66	13	13	13	12	12	14	13
37	47	42	47	52	54	41	46	46	48	56	58	60	58	12	13	13	13	13	15	12
38	48	42	42	50	59	41	64	66	52	56	78	84	70	12	13	13	13	13	15	13
—						+	—						+	—						+
Buffer reference: 50							Buffer reference: 53							Buffer reference: 14						

With K-veronalate/veronal buffer																				
*Potassium**							*Veronal***							*Conductivity****						
—						+	—						+	—						+
52	32	37	39	41	46	48	58	50	56	60	70	84	88	12	9	10	13	12	16	21
53	65	58	48	45	60	51	56	62	57	58	62	84	68	21	22	11	12	12	13	13
52	48	53	47	64	63	47	54	56	52	56	60	80	56	12	15	15	13	12	17	18
40	38	48	51	51	60	46	56	60	60	68	72	76	50	10	16	12	12	17	14	11
38	43	49	46	48	56	53	60	74	80	92	94	76	56	10	11	12	12	12	14	11
—						+	—						+	—						+
Buffer reference: 42							Buffer reference: 62							Buffer reference: 13						

---

* The numbers multiplied by 10^{-3} give concentrations expressed in mequiv./cm^2 paper.

** The numbers multiplied by 10^{-3} give concentrations expressed in mg veronal/cm^2 paper.

*** The numbers multiplied by 10^{-5} give the conductivity of the buffer present in 1 cm^2 and eluted in 1 ml aq. bidest.

U.V., and the conductivity of the eluate itself was measured. These experiments were repeated with reversed polarity in order to eliminate any experimental error caused by the apparatus. Then they were repeated with buffer where *potassium* took the place of *sodium*. The same picture was found in all experiments. The values found are given in Table I, and are represented diagrammatically in Fig. 6.

Results

A. Sodium shows a para-anodic peak.

B. Veronal is to be found in the same region.

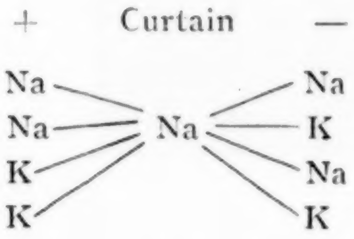
C. The conductivity is increased in the same zone.

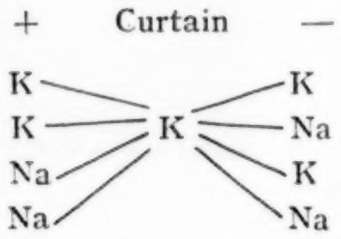
D. The results with potassium are analogous to those with sodium.

E. The limits of any ion shift always have a typical triangular shape pointing towards the center and the bottom of the curtain. The increase of the ionic concentration in the vertical direction is due to an evaporation flow running from top to bottom of the curtain. The increase in the horizontal direction is due to migration in the electrical field.

2. EXPERIMENTS IN WHICH SODIUM AND POTASSIUM BUFFERS WERE USED SIMULTANEOUSLY

To get a better idea of the phenomenon we prepared sodium and potassium buffers of the same ionic strength. Each lateral electrode and the center of the curtain were fed separately by means of individual circuits according to the following general scheme:





The values found are given in Table II. Three diagrams of a complete experiment are given in Fig. 7, and the details of one experiment in Fig. 8.

Results

A. There is no migration of cations in different directions, but merely an accumulation of cation in a para-anodic zone.

B. The various experiments all give analogous results. Thus the observed phenomenon must be considered as representing the normal ionophoretic distribution during curtain electrophoresis.

3. EXPERIMENTS WITH NaLiK BUFFER

To demonstrate these phenomena in one single experiment we devised a much simpler experimental method using three different cations, namely: Na, Li, and K. The values are given in Table III, and the diagram in Fig. 9. It should be noted that the polarity is the reverse of that of the previous experiments.

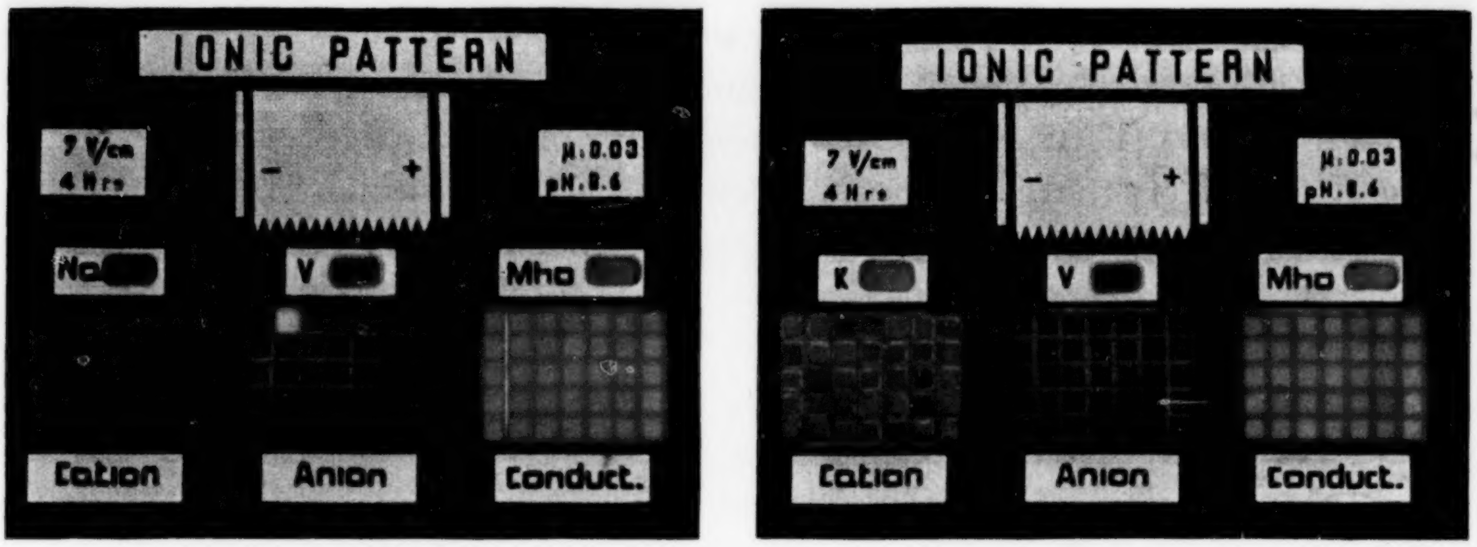

IONIC PATTERN
7 V/cm
4 Hrs
−
+
μ:0.03
pH:8.6
Na
K
V
Mho
Cation
Anion
Conduct.

Fig. 6a Fig. 6b

Fig. 6. Ionic pattern and conductivity in two-dimensional electrophoresis. The colour depth is proportional to the values given in Table I. The small coloured rectangles give the original concentration of the buffer present on the same surface and eluted under the same conditions as the experimental fractions. a. Na-veronalate/veronal buffer. b. K-veronalate/veronal buffer.

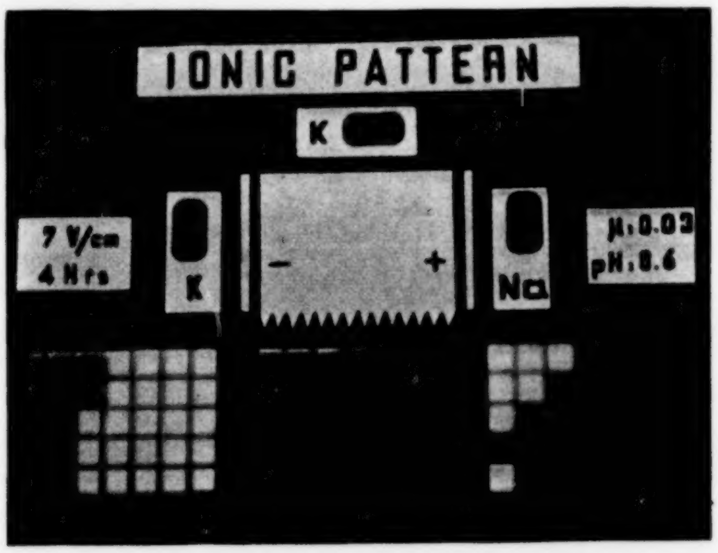

IONIC PATTERN
K
7 V/cm
4 Hrs
K
−
+
Na
μ:0.03
pH:8.6

Fig. 7a

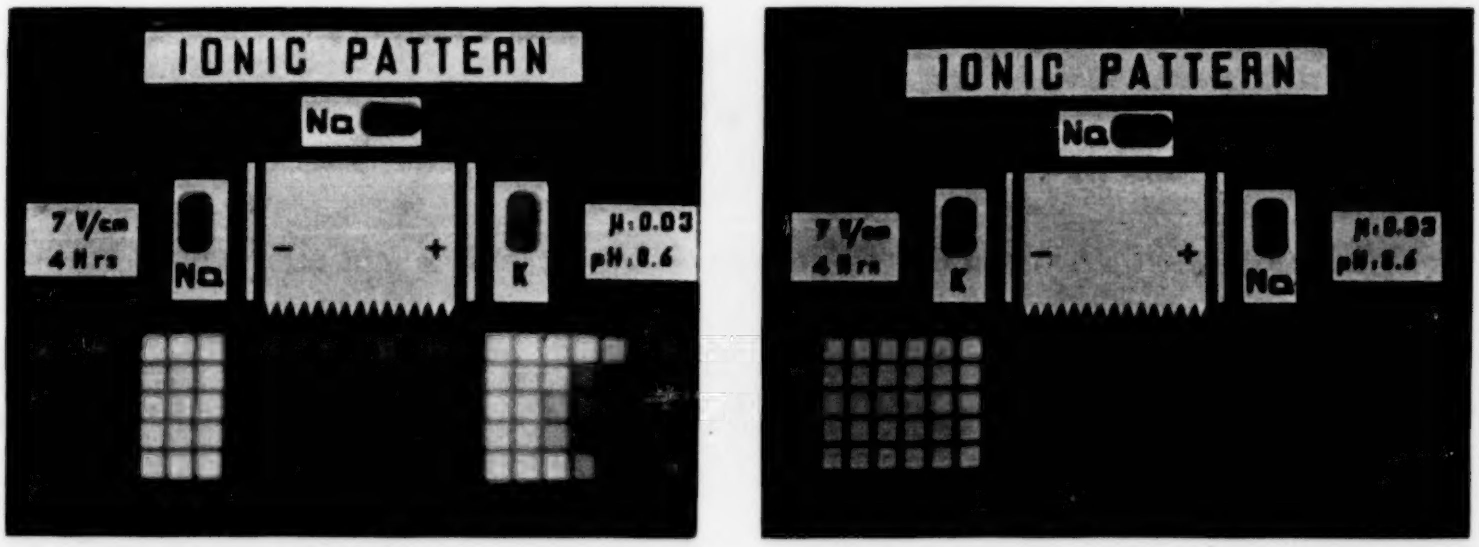

IONIC PATTERN
Na
7 V/cm
4 Hrs
Na
−
+
K
μ:0.03
pH:8.6
IONIC PATTERN
Na
7 V/cm
4 Hrs
K
−
+
Na
μ:0.03
pH:8.6

Fig. 7b Fig. 7c

Fig. 7. Ionic pattern of the cation in two-dimensional electrophoretic fields when sodium and potassium veronalate buffers are used simultaneously. The colour depth is proportional to the values given in Table II. The small coloured rectangles give the original concentration of the buffer present on the same surface and eluted under the same conditions as the experimental fractions. Buffer distribution: a. Cathode: K-veronalate/veronal; Curtain: K-veronalate/veronal; Anode: Na-veronalate/veronal. (For details of this experiment see Figs. 8a, b and c.) b. Cathode: Na-veronalate/veronal; Curtain: Na-veronalate/veronal; Anode: K-veronalate/veronal. c. Cathode: K-veronalate/veronal; Curtain: Na-veronalate/veronal; Anode: Na-veronalate/veronal.

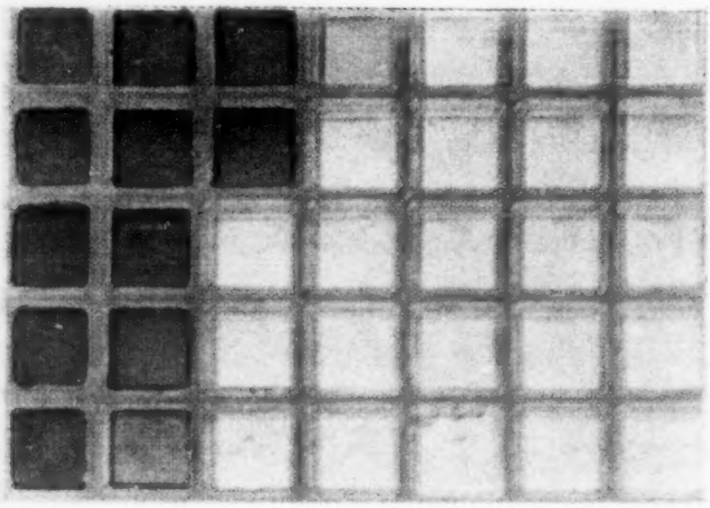


Fig. 8a

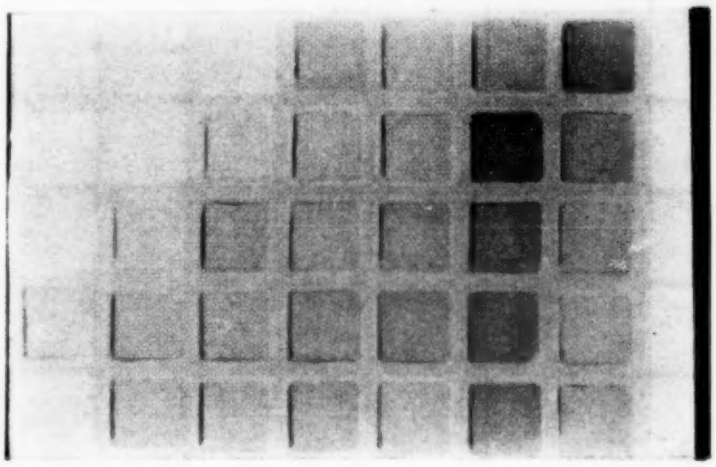


Fig. 8b

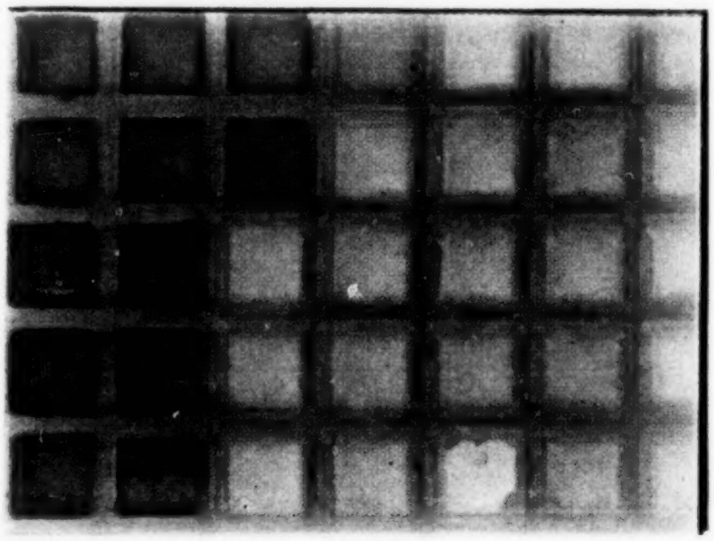
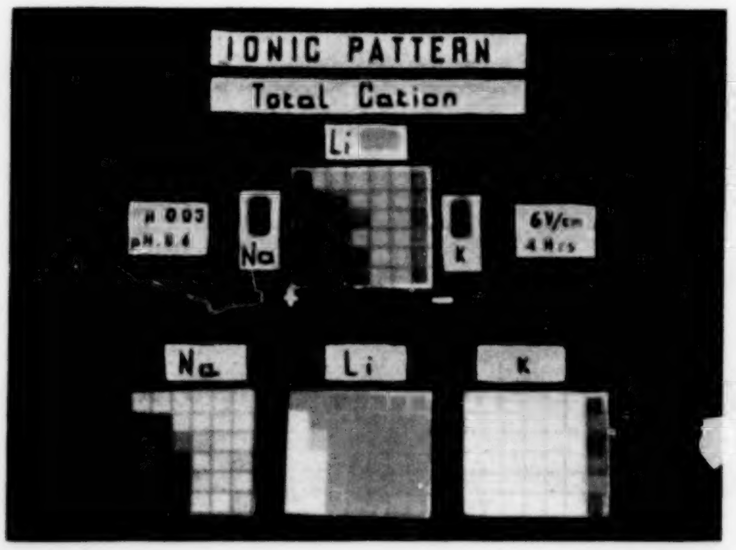


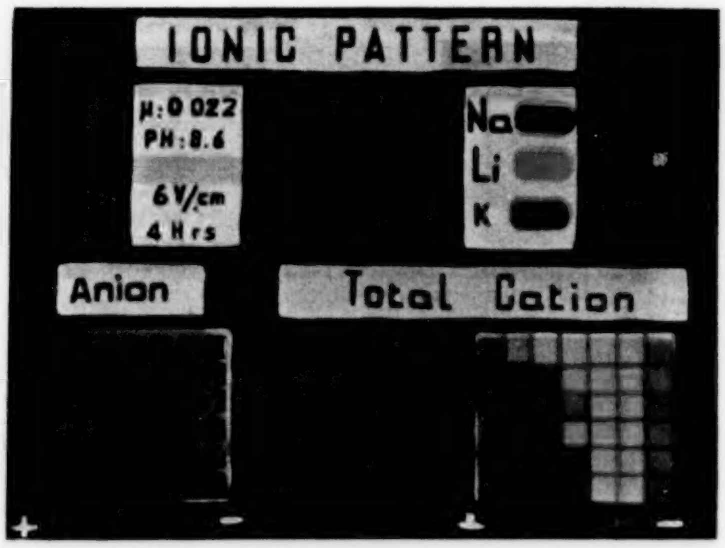
Fig. 8c

Fig. 8. Details of the experiment shown in Fig. 7 a.



IONIC PATTERN
Total Cation
Li
μ 0.03
pH 8.6
Na
K
6V/cm
4 Hrs
+
−
Na
Li
K

Fig. 9a



IONIC PATTERN
μ: 0 022
PH: 8.6
6 V/cm
4 Hrs
Na
Li
K
Anion
Total Cation
+
−
+
−

Fig. 9b

Fig. 9. Ionic pattern in two-dimensional electrophoresis with NaLiK buffer. The colour depth is proportional to the values given in Table III. Buffer distribution: Cathode: K-veronalate/veronal; Curtain: Li-veronalate/veronal; Anode: Na-veronalate/veronal. a. Ionic pattern of the cations. b. Ionic pattern of the anion and total cation in the same experiment.

TABLE II

IONIC PATTERN OF THE CATION IN TWO-DIMENSIONAL ELECTROPHORETIC FIELDS, WHEN SODIUM AND POTASSIUM VERONALATE BUFFERS ARE USED SIMULTANEOUSLY

*Sodium**							*Potassium**							*Total cation**						
*Type of buffer***. Cathode: Na; Curtain: Na; Anode: K (see Fig. 7b)																				
18	18	14	27	0	0	0	1	1	1	7	29	37	38	19	19	15	34	29	37	38
32	40	30	28	0	0	0	1	1	2	31	77	68	72	33	41	32	59	77	68	72
36	38	31	25	0	0	0	1	2	16	40	81	81	63	37	40	47	65	81	81	63
45	54	43	24	0	0	0	1	2	18	36	76	76	60	46	56	61	60	76	76	60
31	45	36	18	0	0	0	1	1	2	29	61	81	40	32	46	38	47	61	81	40
—						+	—						+	—						+
Type of buffer. Cathode: K; Curtain: Na; Anode: Na (see Fig. 7c)																				
39	39	39	43	46	57	68	5	0	0	0	0	0	0	44	39	39	43	46	57	68
61	69	61	57	72	84	61	11	0	0	0	0	0	0	72	69	61	57	72	84	61
45	57	50	45	51	50	74	10	0	0	0	0	0	0	55	57	50	45	51	50	74
72	63	61	64	67	63	54	10	0	0	0	0	0	0	82	63	61	64	67	63	54
52	51	68	63	79	77	67	13	0	0	0	0	0	0	65	51	68	63	79	77	67
—						+	—						+	—						+
Type of buffer. Cathode: K; Curtain: Na; Anode: K																				
35	35	36	33	18	18	18	0	0	1	7	28	29	51	35	35	37	40	46	47	69
33	45	36	18	17	17	17	0	2	11	31	44	43	44	33	47	47	49	61	60	61
36	47	36	18	16	16	16	9	9	22	36	45	50	38	45	56	58	54	61	66	54
36	47	40	16	15	15	15	9	9	22	36	44	49	40	45	56	62	52	59	63	55
35	38	31	16	15	15	15	9	7	14	33	37	40	37	44	45	45	49	52	55	52
—						+	—						+	—						+

(continued on p. 317)

TABLE II (*continued*)

Sodium*							Potassium*							Total cation*						
*Type of buffer***. Cathode: K; Curtain: K; Anode: Na (see Figs. 7a and 8)																				
0	0	0	0	44	53	61	42	44	42	15	0	0	0	42	44	42	15	44	53	61
0	0	26	45	59	67	48	47	48	45	0	0	0	0	47	48	71	45	59	67	48
0	14	36	46	53	58	38	56	48	0	0	0	0	0	56	62	36	46	53	58	38
14	40	48	54	58	64	38	53	38	0	0	0	0	0	67	78	48	54	58	64	38
0	35	43	54	52	63	40	43	24	0	0	0	0	0	43	59	43	54	52	63	40
—						+	—						+	—						+
Type of buffer. Cathode: Na; Curtain: K; Anode: K																				
0	0	0	0	0	0	0	33	41	33	40	41	33	44	33	41	33	40	41	33	44
0	0	0	0	0	0	0	45	45	39	40	48	53	53	45	45	39	40	48	53	53
22	19	18	0	0	0	0	41	45	45	52	52	62	37	63	64	63	52	52	62	37
24	18	18	0	0	0	0	37	45	52	52	62	60	34	61	63	60	52	62	60	34
19	15	14	0	0	0	0	24	41	41	52	45	52	41	43	56	55	52	45	52	41
—						+	—						+	—						+
Type of buffer. Cathode: Na; Curtain: K; Anode: Na																				
22	0	0	34	65	45	58	55	40	16	4.5	4	4	5	77	40	16	39	69	49	63
22	0	36	46	49	63	45	49	62	16	4	4	4.5	4	71	62	52	50	53	68	49
23	25	43	49	52	65	52	38	45	3	3	3	3	3	61	70	46	52	55	68	55
25	32	59	64	56	68	41	36	38	3	3	3	3	3	61	70	62	67	59	71	44
23	25	47	47	54	59	36	41	18	3	3	3	3	3	64	41	50	50	57	62	39
—						+	—						+	—						+

---

[*] Quantities expressed as mequiv./cm^2 × 10^{-5}.

[**] The cathode is always on the left, and the anode on the right.

TABLE III

IONIC PATTERN WHEN Na-Li-K-VERONALATE BUFFERS ARE USED SIMULTANEOUSLY
(see also Fig. 9)

(Quantities expressed as mequiv./cm^2 x10^{-5})

Total cation

Li 90

6 V/cm
4 h

Na 90

K 90

$\mu = 0.022$
pH = 8.6

144	84	93	83	81	86	68
120	162	128	89	84	90	88
111	179	144	99	90	95	93
101	150	141	116	94	99	95
117	135	137	129	94	99	92
135	122	134	143	96	100	89

+ −

Na

65	8					
120	117	60	9			
111	134	77	20	6	5	5
101	150	93	30	12	12	9
117	135	92	45	12	12	9
135	122	92	62	12	12	12

+ −

Li

80	77	93	83	81	86	65
	45	68	80	84	90	86
	23	57	83	83	89	84
		48	86	83	87	83
		45	84	83	87	80
		42	81	84	89	75

+ −

K

						3
						3
						3
						3
						3
						3

+ −

Anion

116	121	100	95	116	96	100
114	145	133	138	110	121	142
117	133	129	144	116	128	156
121	123	128	147	121	133	168
117	140	129	131	124	133	145
112	158	132	116	128	133	121

+ −

INTERPRETATION OF RESULTS

A theoretical explanation for the para-anodic accumulation of electrolytes was given by GUILLOT[8]. The migration velocities of the various ions in solution differ, being greater for the smaller cation than for the organic anion. For one anion discharged at the anode, one cation is discharged at the cathode, while at the same time the entire conductor remains electrically neutral. As the two ions have different migration velocities it is reasonable to assume that the rapid ions are able to cover a larger distance than the slower ones in order to arrive at the point of discharge on the electrode at the same time. As a result the ions accumulate in a zone that lies nearer to the electrode where the slower ions are being discharged. In this case this means a para-anodic ion shift. Owing to the absence of convection this shift of ions inside the paper or other supporting medium is maintained in its actual shape and can be detected after elution of the dried fractions of the curtain. An analogous and characteristic ion pattern was found in zone electrophoresis[7].

The consequences of this ionic shift inside the supporting medium are multiple. As a result of differences in concentration, there are differences in ionic strength and conductivity. This means that the electrical field is not homogeneous over the entire surface.

In a good run the arrival of new buffer, the inevitable evaporation from top to bottom and the ion shift coöperate to maintain a constant ionic pattern throughout the experiment once the conditions are stabilized, *i.e.* 6 hours after closing the chamber and switching on the current.

It is, however, certain that the frequent appearance of daughter fractions coming from a mother fraction at a given point on the curtain is due to this ionic pattern.

The apparent rectilinear migration of albumin, which crosses the zone of increased salt concentration, is due to a balance between slower hydrodynamic flow and a longer acting but lower electrical field in a zone with higher strength and lower mobility.

If the electrophoretic experiment goes wrong, changes in pH will occur as a result of an excessive ion shift on the curtain, appearing as a typical triangular shape forecast by the ionic pattern we have demonstrated.

With the usual sodium barbital buffers this ion shift cannot be avoided, but the use of zwitter-ions may be advantageous in this respect.

We shall repeat the experiments with so-called "NaLiK" buffer and not only measure the quantity of anion and cation, but also determine the quantity of water inside the supporting medium. Then we shall not only be able to give quantities of salt, but also to calculate actual concentrations, ionic strength and pH. We hope that this method of investigating the ionic shift inside the supporting medium will provide a means of improving electrophoretic techniques.

SUMMARY

A study was made of the distribution of ions on a paper curtain during two-dimensional electrophoresis. The influence of evaporation on the one hand and of ionophoresis on the other, was investigated. A characteristic distribution of anion and cation was found to occur, which causes differences in conductivity of the paper.

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[8] M. GUILLOT, *Ann. biol. clin.* (*Paris*), (1956) 1–2, 8.

Received October 6th, 1958

SHORT COMMUNICATIONS

The electrophoretic mobilities of inorganic ions in some inorganic and organic acids

In connection with our previous papers[1] concerning the electrophoretic mobilities of the halogeno-complexes of Hg, Bi, Cd, Pb and Cu, we have made a systematic investigation of the electrophoretic mobilities of about 90 inorganic ions in some inorganic and organic acids. The concentrations of the acids varied from 0.4 to 6.3 *N*.

Some of the mobility data obtained by heavy current and high voltage electrophoresis on filter paper[1] are represented here in diagrams as a function of the logarithm of the concentrations of hydrochloric, perchloric, nitric, sulphuric, acetic, citric, tartaric and lactic acids (Figs. 1 and 2).

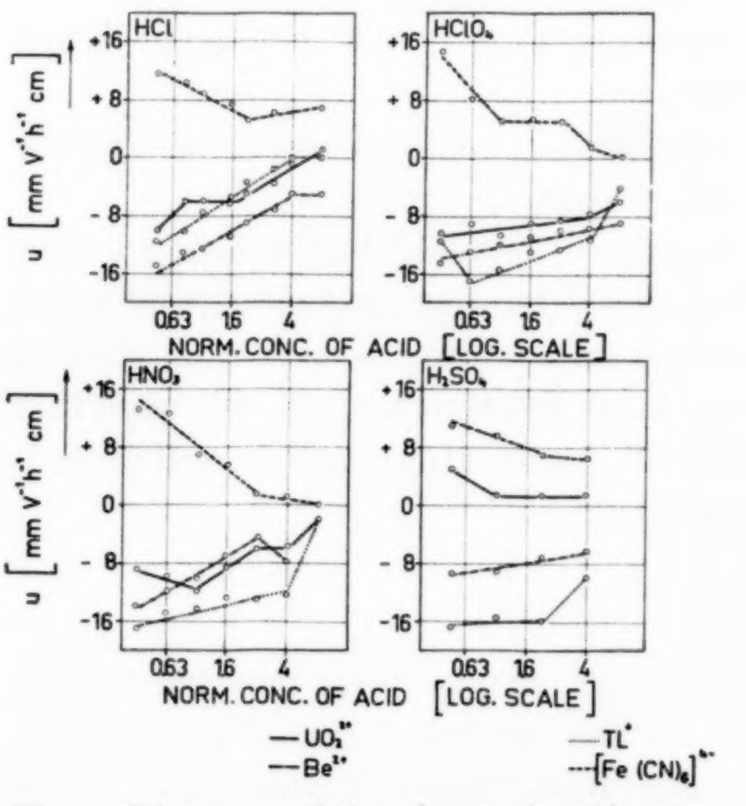

Fig. 1. Diagrams of the electrophoretic mobilities of UO_2^{+2}, Tl^+, Be^{+2} and $[Fe(CN)_6]^{-4}$ in some inorganic acids.

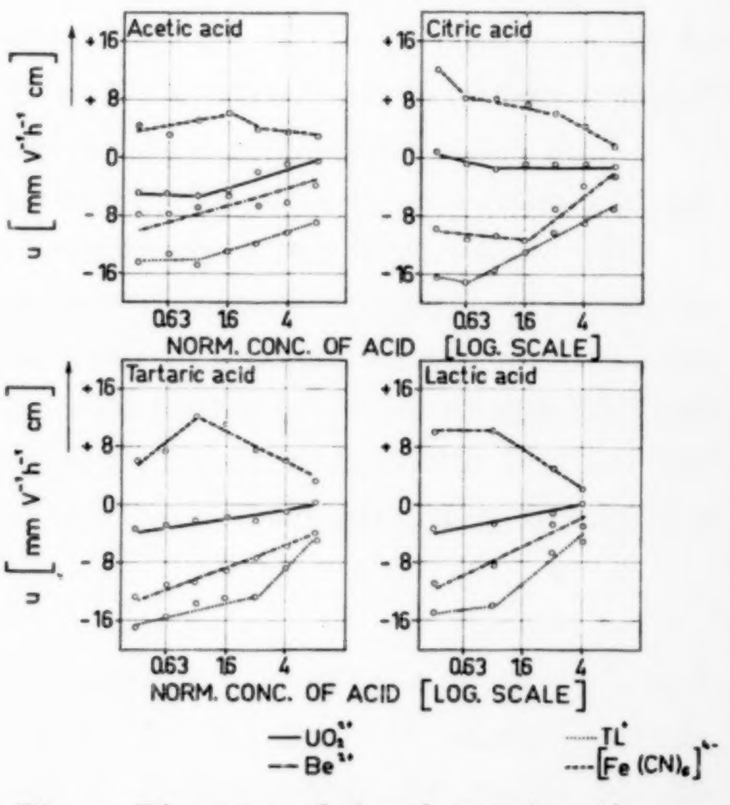

Fig. 2. Diagrams of the electrophoretic mobilities of UO_2^{+2}, Tl^+, Be^{+2} and $[Fe(CN_6]^{-4}$ in some organic acids.

The mobilities of four inorganic ions are reported here: UO_2^{+2}, Tl^+, Bi^{+2} and $[Fe(CN)_6]^{-4}$. The filter paper used was Munktell No. 20/100. The mobility data were corrected according to the equation[1]:

$$u = 1.2\left(\frac{u'}{R_F} - \frac{u'_e}{R_{Fe}}\right) [\text{mm V}^{-1}\ \text{h}^{-1}\ \text{cm}]$$

where u' is the apparent mobility of the ion investigated, u'_e the apparent electro-osmotic mobility, R_F the adsorption chromatographic factor on filter paper of the ion investigated, R_{Fe} the adsorption chromatographic factor of the substance used to determine the electro-osmotic flow (glucose), and 1.2 is the correction factor for the porosity and structure of the filter paper used. The apparent shift of the spots is measured in millimeters (mm), the apparent electric field strength in volts per centimeter (V/cm), and the time in hours (h). The resulting mobilities [mm V^{-1} h^{-1} cm] can be transformed to [cm^2 V^{-1} sec^{-1}] by multiplying them with the factor $0.278 \cdot 10^{-4}$.

The diagrams in Figs. 1 and 2 show characteristic slopes, breaks and parallelisms.

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Paper chromatographic reagents in aerosol spray bottles

During the last few years numerous cosmetic products have been marketed in aerosol bottles that are fitted with a nozzle and stop valve, by means of which the contents are dispensed as a fine spray. Low boiling halogenated hydrocarbons are generally used as propellants. These are present in the bottle in the liquid state under a certain pressure. For practical purposes the stop valve and nozzle are constructed as a unit that can be operated by pressing one finger on the nozzle. The author thought that this arrangement might be suitable for spraying chromatograms.

An experiment was carried out with a solution of *p*-anisidine phosphate in ethanol. This reagent, which is usually employed for the detection of sugars on chromatograms, was filled into an aerosol bottle together with the propellant. The bottle was made of aluminium and was covered inside with an epoxy protective. Some chromatograms were sprayed with the bottle and others, for comparison, with an ordinary glass sprayer. The advantages of the aerosol bottle were a more uniform distribution of the reagent and simpler handling, since only one finger need be used instead of the two hands required to manipulate an ordinary glass spraying apparatus. Another advantage of the aerosol bottle is that it is always ready for use and need not be cleaned after use.

Of course, there may be limitations to its use. It may be dangerous, for example, to use it with alkalies in ethanol (risk of injuries to eyes, etc.). Corrosion of the bottle may also occur with some reagents; however, materials resistant to most chemicals are already known. From the manufacturing point of view, the rapid evolution of paper chromatography, giving rise to a continual change of reagents, may discourage chemical dealers from launching such areosol bottles on the market. However, this does not apply, for example, to the ninhydrin reagent. It is felt that the introduction of aerosol bottles could be promoted by research institutes and industrial laboratories and the author would be grateful for encouragement in this direction.

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Klaus Wegmann

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Ionization chamber for high-temperature gas chromatography

Simultaneous monitoring of the effluent stream from gas chromatography equipment containing radioactive compounds by an ion chamber in series with the usual mass-detecting thermal-conductivity cell has been described by WILZBACH AND RIESZ[1]. However, when this design of ion chamber employing a ceramic insulator was used at 200° to 250°, as required for the gas chromatography of fatty acid esters, strain currents limited its applicability.

Construction of an ionization chamber from readily available materials is described which is applicable to high-temperature (240°) gas chromatography.

A successful model, illustrated in Fig. 1, based upon the use of Teflon, was evolved after studying a number of potentially useful high dielectric constant materials. Teflon comprises the main insulator *a*, which is threaded to receive the brass can *l*, support plugs *b* and *c*, and inlet and outlet connectors *d* and *e*. The ion chamber *l* has an internal working volume of about 9 ml.

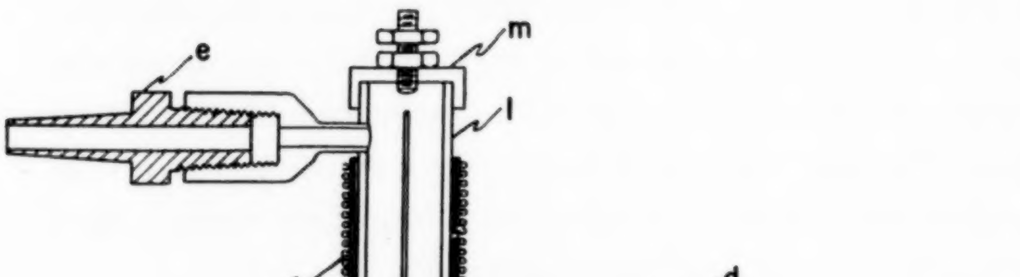

Fig. 1. Ionization chamber.

The probe *n* is constructed from BSA-18 gauge tungsten rod and is connected to the brass contact pin through a short piece of copper wire silver-soldered to the probe and soft-soldered to the contact pin. A hole is drilled in the top of an Amphenol 82–833 fitting *f* and closed with brass plug *g*. Internal parts of the fitting, except the polystyrene insulator *h* and its brass contact pin, are removed. The modified fitting is connected to the preamplifier head of a Brown Electrometer through the coaxial "cable" consisting of a threaded brass tube *i*, a brass rod *k*, and plug *c*. A current of air is used to cool the tube to prevent heat transfer from the chamber to the preamplifier. Brass cap *m* is provided with a bolt and nuts for connection to the negative side of a 4.5-volt battery. The positive side is grounded on the preamplifier case.

Around the ion chamber *l* is wrapped a layer of asbestos *p*, a grounded metal shield *q*, and a layer of No. 26 asbestos-covered nichrome wire *r*. Movement of insulator *a* relative to a brass shield, which screws into the coaxial fitting, is prevented by set screw *o*.

In operation the chamber is padded with Pyrex wool and covered with a grounded metal shield containing the necessary exit holes for gas streams and electrical leads. This shield eliminates capacity effects and lessens heat loss. Pyrex wool and asbestos paper are used to insulate the standard taper inlet connector *e* after it is plugged into the exit connector from the thermal conductivity gauge of the chromatography equipment, *i.e.*, Aerograph. Current is supplied to the heater wiring with a variable autotransformer. The temperature is adjusted by temporary insertion of a thermometer in place of connector *d*.

Fig. 2 illustrates results obtained with about 2 mg (3 μC) of tritium labeled methyl stearate[2] using a 5-foot Resoflex 296 column[3] operated at 205° and at a helium flow rate of 44 ml/min. The thermal conductivity data are recorded at 2 mV, full-scale sensitivity, and ion currents are recorded using the 10 mV range of the electrometer. Both recorder charts were run at 60 inches per minute and are superimposed in Fig. 2. At the specific flow rate used in this equipment, radioactivity lags thermal conductivity by about 20 seconds. These curves demonstrate the chromatographic identity of tritium labeled stearate with inactive stearate and indicate a small amount of labile tritium contaminant.

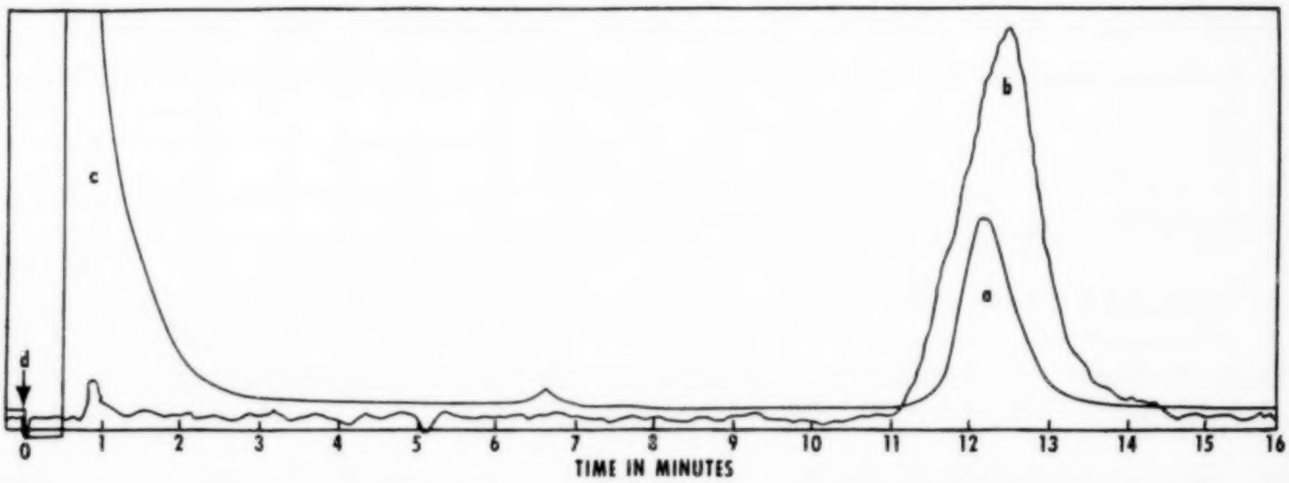

Fig. 2. Gas chromatography of tritiated methyl stearate. a. Thermal conductivity peak. b. Radioactivity (ion current) peak. c. Solvent peak. d. Point of injection.

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Identification of certain anions and cations in mixtures by means of the formation of coloured (ring) products on agar gel

Part 1. Identification of anions and cations on gel containing the test reagent

Introduction. When a suitable reagent is added to a solution containing two ions both of which react with the reagent, the identification of the ions is not possible. Some work has already been done on the detection of ions in mixtures by using filter-paper strips impregnated with agar gel containing the detecting reagent$^{1-3}$. This paper describes a simple method for detecting such ions, without the use of any elaborate apparatus, by means of the formation of coloured rings on agar gel supported on a horizontal glass plate. The relative position of the coloured rings has been found to be dependent on the solubility of the products involved, the less soluble product forming the inner ring.

Experimental. Agar, B.D.H., L.R. grade (fine powder) was used in the experiments. It was found to be free from all ions except negligible traces of chloride ions.

TABLE I

Sample No.	*Ions contained in the mixture*	*Detecting reagent*	*Inner ring*		*Outer ring*	
			Colour	*Reacting ion*	*Colour*	*Reacting ion*
	Anions					
1	Ferrocyanide and thiocyanate	Ferric chloride	Blue	Ferrocyanide	Red	Thiocyanate
2	Ferrocyanide and ferricyanide	Copper sulphate	Chocolate	Ferrocyanide	Yellow	Ferricyanide
3	Ferricyanide and ferrocyanide	Silver nitrate	Orange	Ferricyanide	White	Ferrocyanide
4	Iodide and ferricyanide	Silver nitrate	Yellow	Iodide	Orange	Ferricyanide
5	Iodide and chloride	Silver nitrate	Yellow	Iodide	White	Chloride
6	Sulphide and iodide	Mercuric chloride	Black	Sulphide	Orange	Iodide
	Cations					
1	Nickel and cobalt (as complex ammines)	Rubeanic acid	Blue	Nickel	Yellow-brown	Cobalt
2	Ferric and titanic	Cupferron	Red	Ferric	Yellow	Titanic
3	Ferric and titanic	Potassium ferrocyanide	Blue	Ferric	Green	Titanous
4	Ferric and manganous	Potassium ferrocyanide	Blue	Ferric	White	Manganous
5	Copper and cadmium	Potassium ferrocyanide	Chocolate	Copper	White	Cadmium
6	Lead and silver	Potassium chromate	Yellow	Lead	Red	Silver
7	Thorium and uranyl	Alizarin sulphonic acid	Pink	Thorium	Bluish violet	Uranyl

1% agar gel was prepared by boiling agar for a few minutes with water and the concentration of the reagent incorporated in the gel was adjusted to about $N/100$. Test solutions contained equal volumes of $N/2$–$N/20$ solutions of each ion. A thin layer of the gel containing the reagent was allowed to spread on a glass plate and one drop (0.05 ml) of the test solution was placed at the centre of the gel before it was allowed to set. It was observed that distinct rings for different ions were obtained within 2 to 4 hours at room temperature (about 25°). The results are given in Table I.

Part 2. Identification of certain anions on gel containing silver chromate

Introduction. Filterpaper impregnated with silver chromate has been used for detection of halide ions[4]. In the present investigation finely dispersed silver chromate in agar gel has been employed and a separation of halides and other anions has been obtained.

Experimental. A fine dispersion of silver chromate in agar gel was obtained as follows: to 80 ml of hot 1% agar gel were added 10 ml of $N/10$ silver nitrate solution followed by a slight excess of $N/10$ potassium chromate solution. The procedure for detection of the anions was the same as adopted in Part 1; the results are given in Table II.

TABLE II

Sample No.	*Anions contained in the mixture*	*Inner ring*		*Outer ring*	
		Colour	*Reacting ion*	*Colour*	*Reacting ion*
1	Sulphide and chloride	Black	Sulphide	White	Chloride
2	Iodide and sulphide	Yellow	Iodide	Black	Sulphide
3	Iodide and ferricyanide	Yellow	Iodide	Orange	Ferricyanide
4	Iodide and arsenate	Yellow	Iodide	Chocolate	Arsenate

Conclusions

This investigation provides a simple method of detecting certain anions and cations in mixtures when they are present in quantities of the order of 2 mg per ml, which normally require more elaborate methods. The coloured rings for various ions can readily be obtained in a state of transparency and, with suitable experimental modifications, they may be employed for colorimetric estimations. The above technique also provides a method for determining the comparative solubilities of insoluble substances.

The authors express their sincere thanks to Prof. T. R. SESHADRI, Head of the Department of Chemistry, for helpful suggestions and for his keen interest in this work.

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Paper electrophoresis of inorganic anions in 0.1*N* NaOH solution

Extensive work on the electromigration of inorganic anions has so far only been reported for KCl[1] and ammonium bicarbonate[2,3] as electrolytes. Recently JACH *et al.*[4] employed 0.01 *N* NaOH as electrolyte for the separation of anion mixtures obtained in Szilard-Chalmers reactions. They showed that such mixtures as BrO_3^-–Br^-, IO_4^-–IO_3^-–I^- and AsO_3^{---}–AsO_4^{---} yielded good separations. We thus considered it of interest to study a large number of the usual inorganic anions with NaOH as electrolyte. Instead of 0.01 *N* NaOH we used 0.1 *N* NaOH as in a higher concentration more material may be separated without distortion due to increased conductivity of the spots. The large electrophoresis apparatus of Jouan was employed and a thick paper (Papeterie d'Arches No. 304). The spots were placed exactly in the centre of the sheet and a voltage of 240 V was applied for 1 hour. Under these conditions CrO_4^{--} moves approximately 80 mm. As it is easily recognised by its colour and has an intermediate mobility all other distances moved were corrected to CrO_4^{--} 80 mm. Periodate yields usually one or two spots close to the point of application and a reduction comet up to the distance moved by iodate. The movement of the other ions examined is reported in Table I.

TABLE I

ELECTROMIGRATION OF INORGANIC ANIONS IN 0.1 *N* NaOH IN ONE HOUR WITH 240 V

The distances are corrected from one electropherogram to the next to a movement of 80 mm for CrO_4^{--}

Anion	*mm*	*Anion*	*mm*
Borate	30–32	Thiocyanate	72–77
Arsenite	23–27	Sulphite	62–69
Arsenate	51–54	Thiosulphate	86–89
Nitrite	92	Sulphate	73–81
Nitrate	81–84	Persulphate	85
Chloride	90–91	Selenite	58–60
Chlorate	72–76	Tellurite	49–52
Perchlorate	73–80	Tellurate	0
Bromide	92–96	Ferrocyanide	73–77
Bromate	60–65	Ferricyanide	79–80
Iodide	96–101	Molybdate	69–74
Iodate	40–42	H_2O_2	0
Periodate	5–15 with comet to 40	Orthophosphate	46–48
Fluoride	60–61		

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Received December 10th, 1958

Paper chromatographic front indicators

On carrying out paper chromatography with sheets of paper it is found that solvent fronts are never straight.

MÜNZ[1] explained in detail why solvent fronts run in an uneven manner on sheets of smooth filter paper rolled up into a cylinder. In most cases conditions of steady run cannot be maintained, and this results in uneven solvent fronts which are rather difficult to mark. Similarly, difficulties are encountered when attempting to mark solvent fronts in the case of volatile solvents. To facilitate the technique of paper chromatography it therefore seems necessary to evolve a method for marking front lines.

In the present experiments, the behaviour of 31 dyes in different solvents was examined. The dyes tested consisted of various types (phenazine, azo, triphenylmethane, indigoid, xanthene, thiazine, phthaleine) that are often applied in most laboratories. Most of the dyes were used in aqueous solution; those that are insoluble in water were dissolved in 96% ethanol. Of the usual solvents employed in chromatography, only twelve that are most often applied, were selected. Experiments were carried out with Whatman No. 1 filter paper, but sometimes also replicate determinations were made with Schleicher and Schüll 2043 b type filter paper, to find out whether there are differences between the two papers when they are tested with the same dyes in the same solvent.

When the ascending technique was applied the dyes were transferred to paper sheets of 25 × 21 cm, on a "starting line" 2.5 cm from the top of the paper. Lines 1 cm long were drawn with each dye by means of a thin glass capillary, leaving free spaces of 0.25 cm between consecutive lines. After rolling up the sheets, chromatograms were run in two large beakers with ground edges, one of which was inverted and placed on top of the other. The beakers were closed airtight by applying a vaseline film to their ground edges. Chromatograms were run in a thermostat at 23–25°.

When using the descending technique, sheets of 25 × 25 cm of smooth filter paper were employed, and the starting line of the dyes was located 2.5 cm from the bottom of the sheet.

The dye concentrations varied between 0.01 and 1.0%, according to the intensity of the colour.

It is extremely important that the dye solution, on leaving the thin capillary, should form a stripe not wider than 1–3 mm when the front line is being marked with a ruler.

Running was stopped when the solvent front had passed 1.0 to 1.5 cm beyond the starting line of the dyes. After removing the solvent, the front line should be marked by pencil, because the dye line may become diffuse (indistinct) or may migrate during spraying with the developing reagent or, in two-dimensional chromatography, when a second solvent is applied.

In practice, it proved advantageous to draw the dye line 0.5–1.0 cm before the place where the solvent front was expected to arrive. In the case of a rather straight

TABLE I

Dye	*Whatman No. 1*												*S & S 2043 b*	
	Solvent												*Solvent*	
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*k*	*l*	*m*	*c*	*d*
Safranine TH									++					+
Baumwollrot 4 BC														
Bismarck Brown				+		+		++	++					+
Crystal Violet	++	+	+		++	+	++		++	+	++	++	++	+
Erioglaucine A					+									
Auramine O					++	+	++		++		+	+		
Orange II					++									
Rhodamine GGH	++	++	++	+	++		++	++	++	+	++	++	++	++
Metanil Yellow					++	+								
Methylene Blue														
Direct Green B						++								
Solarflavine 5 G						+								
Rhodamine ZS	+	+	+		++	+	+	+	++	+	++	++	+	
Erythrosin	++	++	++	+	++				++			++	++	+
Indigocarmine														
Rosaniline					++		+	+	++					
Nigrosine, ethanol-soluble	++	++	++	+		++	++	++	++	++	++	++		++
Nigrosine, water-soluble						++							++	
Bromocresol Green					++	+								
Bromocresol Red					++	++								
Fuchsin	+	+			++	+	++	+	++			+	++	
Malachite Green	+	+		++	++	++	++	++	++	++	++	++	++	++
Methyl Blue					+									
Eosin-Na	++	++	++		++	+			+			+	++	
Fluorescein-Na	++	+	+	+	++		+						+	+
Sudan III	++	++	++	++			++	++	++	+	+	++	++	++
Litmus														
Lacmoid						++								
Methyl Red	+	+	++	+			+		++				++	+
Methyl Green									++					
Direct Turkish Blue														

++ = extremely sharp front line, + = less sharp front line.

solvent front, even less suitable dyes yield sharp lines after migrations of 0.5 cm. The 0.5–1.0 cm run of the dyes corresponds even in short-distance runs (20 cm) to the R_F interval of 0.95–1.00, so that there is little risk of the dyes causing interferences, since only a few substances with R_F values exceeding 0.95 are known.

The behaviour of the dyes in various solvents is summarized in Table I.

The solvents applied were the following:

(a) Butanol–acetic acid–water (4:1:1).

(b) Butanol–acetic acid–water (4:1:5). In solvent mixtures (a) and (b) suitable dyes partly remained at the site of application, Sudan III and Methyl Red giving the smallest residual spots.

(c) Ethyl acetate–acetic acid–water (3:1:1). Erythrosin and Sudan III completely disappeared from the starting line.

(d) Tetrahydrofuran–water (3:2). Malachite Green, Sudan III and Methyl Red gave no spots at the starting line.

(e) Acetone–water (3:2). Sharp front lines without residual spots were given by Auramine O, Orange II, Rhodamine GGH, Metanil Yellow, Erythrosin, Eosin-Na, Fluorescein-Na and Bromocresol Red. The other suitable dyes left small residual spots at the starting line.

(f) Pyridine–water (13:7). Auramine O, Metanil Yellow, Rhodamine ZS, Bromocresol Green, Bromocresol Red, water-soluble Nigrosine, Malachite Green and Lacmoid proved to run without leaving residual spots.

(g) Butanol–ethanol–water (5:1:4). All the suitable dyes left residual spots at the starting line.

(h) Collidine, saturated with water. Only Sudan III proved to run without leaving a residual spot, other dyes gave small spots.

(i) Phenol, saturated with water. Of the suitable dyes, Bismarck Brown, Rhodamine ZS, Fuchsin and Malachite Green gave very small spots.

(k) Amyl alcohol, saturated with water. All suitable dyes gave appreciable spots at the starting line. In this case the solvent front should not pass more than 1 cm beyond the starting line.

(l) Butanol–3% ammonia. All dyes yielding sharp front lines left appreciable residual spots at the starting line. The dyes behaved as if they were composed of two ingredients, one of them remaining at the starting line, the other migrating with the solvent front.

(m) Butanol–formic acid–water (77:10:13). Only Erythrosin proved to run without leaving a residual spot, all the other dyes gave appreciable residual spots at the starting line.

It was our general experience that with all solvents tested the yellow dyes (Auramine O, Metanil Yellow, Solarflavine 5 G, Fluorescein-Na) gave sharp lines of satisfactory strength only in high concentrations. In the case of lower dye concentrations, the solvent front can be marked by transillumination.

The migration of the dyes with the solvent front was practically the same in solvent systems of identical components but of various concentrations.

Our experiments proved that, on using Whatman No. 1 filter paper with both the ascending and the descending technique, certain dyes (such as Crystal Violet, Rhodamine GGH, Rhodamine ZS, ethanol-soluble Nigrosine, Malachite Green, Sudan III) are suitable for marking front lines of nearly all the solvents used in paper chromatography.

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BOOK REVIEWS

Gas Chromatography (A Symposium held under the auspices of the Analysis Instrumentation Division of the Instrument Society of America, August 1957), edited by V. J. COATES, H. J. NOEBELS AND I. S. FAGERSON. Published by Academic Press Inc., New York, July 1958, 313 pp. Price $ 10.00; after October 1st, 1958, $ 12.00.

In a paper given at this Symposium, A. J. P. MARTIN pointed out: "the present position of gas chromatography... seems to be the rapid application of well-known methods to an infinity of problems", and the other 26 papers amply supported this statement. It is extremely valuable to hold formal and informal discussions between experts and beginners and also persons who may have only just realised the great potentialities of Gas Chromatography. This book provides a record of the papers presented and the formal discussions after each paper.

The papers can be divided into five main groups:

(i) Considerations of the Theory of Gas Chromatography,
(ii) The Application of Gas Chromatography Instrumentation in the Laboratory,
(iii) The Analysis of High Boiling Materials by Gas Chromatography,
(iv) The Application of Gas Chromatography to the Purification of Chemicals, and
(v) Gas Chromatography Instrumentation for Continuous Automatic Analysis.

Three papers in particular may be mentioned for their probable influence in the future. The first by GOLAY is entitled "Theory and Practice of Gas-Liquid Partition Chromatography with Coated Capillaries". The technique described will probably become widely used for the achievement of very high resolution or very short analysis time, provided that a small sample and a sensitive detector are used. The second paper by PHILLIPS on "Gas Chromatography Instrumentation for the Laboratory" describes the use of metal salts, *e.g.* zinc stearate, as column liquids. Highly specific retardation of solutes can be achieved with these metal salts and this principle can be applied to many analytical problems. These studies should be of interest as a model for work at very high column temperatures (above 500°) for which purely inorganic metal salts may soon be used as column liquids. The third paper by FELTON on "A Novel High Temperature Gas Chromatography Unit" describes a simple and low-cost apparatus which can be operated under vacuum or pressure at over 500°, and which should be extremely useful for the analysis of high boiling liquids and solids.

This book has a comprehensive bibliography of 442 references to the middle of 1957, but it is unfortunate that in the interval between the completion of the bibliography and the present date there are now double the number of references. The book contains some considerations and recommendations on Standard Nomenclature for Gas Chromatography, and has author and subject indexes, the latter of which is too rarely found in books of this type.

The printing and illustrations are of a good standard but there are a number of small errors which should have been corrected at an early stage, *e.g.* p. ix, gas-lipid; p. 30, ref. 11, Grob not Grab; etc.

The book can be recommended not only to specialists for its useful details of new developments but also to beginners who will benefit from careful reading of many of its papers.

C. J. HARDY (Harwell)

La chromatographie, par L. SAVIDAN. Monographies Dunod, Paris, 1958, 109 pages, 32 figs., 70 références.

Cette petite introduction à la chromatographie débute par un exposé théorique relativement important (¼ de l'ouvrage), suivi d'une description succinte des procédés de partage sur colonne et sur papier, ainsi que des appareils nécessaires.

Les applications de la chromatographie en chimie organique et en chimie minérale sont ensuite effleurées dans deux courts chapitres (contenant 45 références bibliographiques au total).

Cet ouvrage est trop peu documenté pour être utile au praticien. Seuls, des étudiants désirant s'initier aux principes de la chromatographie tireront parti de sa lecture. On peut d'ailleurs regretter que l'auteur n'ait pas songé à indiquer, pour ceux de ses lecteurs qui désireraient continuer plus avant l'étude des techniques chromatographiques, quels sont les traités complets qui font actuellement autorité dans ce domaine.

E. D.

NEW BOOKS

Preparation of Single Crystals, by W. D. LAWSON AND S. NIELSON. (Butterworths, London, and Academic Press, Inc., New York, 1958), 255 pages, price 45 s.

Steric Effects in Conjugated Systems, edited by G. W. GRAY. Proceedings of a symposium held at the University, Hull, 15–17th July 1958, by the Chemical Society. (Butterworths, London, and Academic Press, Inc., New York, 1958), price 30 s.

Chromatographie mit Aluminiumoxyden "Woelm" (Woelm-Mitteilungen AL7, available from M. WOELM, Eschwege, 2nd edition, 1958), 24 pages. German or English. Contains fifteen examples for the chromatography with alumina. 30 selected references.

Eletroforese em papel e métodos relacionados, by L. P. RIBEIRO, E. MITIDIERI AND O. R. AFFONSO. (Servico Gráfico do I.B.G.E., Rio de Janeiro, 1958), 553 pages, 130 figures, 42 tables and 2547 references.

Gas Chromatography 1958. Proceedings of the Second Symposium organised by the Gas Chromatography Discussion Group under the auspices of the Hydrocarbon Research Group of the Institute of Petroleum and the Koninklijke Nederlandse Chemische Vereniging, held at the Royal Tropical Institute, Amsterdam, 19–23 May 1958. (Edited by D. H. DESTY. Butterworths, London), 383 pages, price 70 s.

Announcement

CHROMATOGRAPHIE DES GAZ – GROUPE DE STANDARDISATION

Sous l'égide de la section de Chimie Analytique de l'Union Internationale de Chimie Pure et Appliquée, un groupe a été constitué en vue de présenter au Conseil de l'Union des propositions concernant la *standardisation de la nomenclature et de la présentation des données de Chromatographie des gaz.*

Ce groupe est présidé par Mr. D. AMBROSE (National Chemical Laboratory, Teddington, Grande Bretagne). Il comprend Messieurs: Dr. A. T. JAMES (Grande Bretagne), Prof. Dr. A. I. M. KEULEMANS (Pays-Bas), Dr. E. KOVATS (Suisse), Dr. R. RÖCK (Allemagne), Dr. F. H. STROSS (U.S.A.) et C. ROUIT, Ingr.-Dr. (France).

Le travail actuel du groupe consiste à mettre au point un document basé sur deux articles parus dans *Analytical Chemistry*, Vol. 30, No. 10, octobre 1958:

p. 1582: "Presentation of Gas–Liquid Chromatographic Retention Data", par D. AMBROSE, A. I. M. KEULEMANS ET H. H. PURNELL.

p. 1586: "Terms and Units in Gas Chromatography", par H. W. JOHNSON ET F. H. STROSS.

En vue de rendre aussi ouverte et aussi large que possible la discussion qui conduira à la publication du document envisagé, toutes les personnes qui utilisent la chromatographie des gaz ou travaillent à en développer les théories et les applications sont invitées à collaborer avec le groupe.

A cet effet, leurs remarques, critiques, suggestions ou approbations concernant les données des publications sous référence pourraient être adressées à:

Monsieur CHARLES ROUIT
Chef du Département Laboratoires
Raffinerie de la Société Française des Pétroles BP
LAVÉRA-MARTIGUES (Bouches-du-Rhône, France)

L'étape initiale conduira à un document en anglais. Un document identique en français est en préparation et sera proposé en temps utile pour discussion aux intéressés.

EDITORIAL

Compared to Volume One of the Journal of Chromatography the number of papers received during the last year has about doubled. Since it is part of the editorial policy to publish short communications and original papers as quickly as possible, it has been arranged to enlarge the current volume slightly, in order to keep the delay in publication reasonably short.

This temporary measure however will be insufficient in the future. It has thus become necessary to plan two volumes of the Journal of Chromatography for the year 1960, of about 550 pages each, appearing in monthly issues.

The number of review articles per year will remain the same as in the past, hence per volume about half the present. The data section will be continued and will contain a novel feature: original R_F values have been offered from several laboratories and where no further description of technique is necessary these will appear in the data section.

As, due to administrative problems, the new arrangement can not be started till 1960, for one or two issues the delay of publication may be slightly more than the maximum of six months generally allowed, but will then decrease to about three or four months early next year and it is hoped to keep it there permanently.

Paris, May 1959 M. LEDERER

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ADSORPTIONS- UND FÄLLUNGSCHROMATOGRAPHIE ANORGANISCHER VERBINDUNGEN

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EINLEITUNG

Im Gebiet der chromatographischen Trennung, des Nachweises und der Bestimmung anorganischer Verbindungen werden zwei Methoden vielfach und mit bestem Erfolg angewendet: Die Austauschchromatographie und die Verteilungschromatographie.

Die Grenzen dieser beiden Methoden sind wegen der Austauschereigenschaften der Cellulose in der praktischen Ausführung nicht immer klar zu ziehen. Noch mehr gilt dies aber gegenüber einer dritten Art der Durchführung chromatographischer Versuche, welche entsprechend der in der organischen Chemie so viel verwendeten und dort allgemein auf "Adsorption" zurückgeführten Methode auch in der anorganischen Chromatographie als Adsorptionsmethode bezeichnet wird. Dies, obwohl unter den dabei herrschenden Umständen Vorgänge anderer Art als bei der Verwendung organischer Substanzen, nämlich Austauschreaktionen, vor allem aber auch Fällungsvorgänge eine grosse Rolle spielen können.

Im Folgenden soll über die Entwicklung auf diesem Gebiet ausführlich berichtet, und Austausch- und Verteilungschromatographie nur soweit berührt werden, als sie strittige oder Grenzfälle betreffen. Solche liegen gegenüber der Verteilungschromatographie z.B. vor, wenn ein wasserlösliches Lösungsmittel wie Methanol im wässrigen System verwendet wird, wobei es als Adsorptionsschichte auf Papier wie eine zweite flüssige Phase wirken kann (Vgl. unten A.2). Über die Abgrenzung gegen die Austauschchromatographie siehe unter A.6.

A. DEFINITIONEN

In der Literatur über das Gebiet werden manche Begriffe in verschiedener oder misszuverstehender Weise verwendet, sie müssen daher, insbesonders im Verhältnis zu den Begriffen der organischen Chemie, eindeutig definiert werden.

1. Es scheint zunächst sogar notwendig den Begriff "Chromatographischer Vorgang" näher zu bestimmen, nämlich als eine Abtrennung wenigstens eines Bestandteiles aus einer Lösung, durch Fixierung in einer Zone auf der Oberfläche eines Festkörpers aus der ihn in einer konstanten Richtung umströmenden Flüssigkeit.

2. Unter "Adsorption" wird im Folgenden die Fixierung einer gelösten Substanz an einer Oberfläche verstanden, ohne Rücksicht auf den Mechanismus des Vorganges.

Es kann spezieller unterschieden werden zwischen Molekel-Adsorption, Ionen-Adsorption (Anlagerung von Ionen an entgegengesetzt geladenen Stellen des Adsorbens), Austausch-Adsorption (Verdrängung eines Ions des Adsorbens durch eines der Lösung) und Fällungsadsorption (durch Reaktion von gelösten Teilen des Adsorbens mit Lösungsbestandteilen unmittelbar an der Oberfläche entstandene und dort fixierte feste Phasen).

Es kann auch bei Verwendung *einer* Lösungsmittelphase der Fall eintreten, dass die Oberfläche des Adsorbens in zunehmendem Ausmass von einer Molekelschichte bedeckt wird, welche nicht der Zusammensetzung des homogenen Lösungsmittels entspricht, wenn dieses ein Gemisch darstellt, z.B. Wasser–Methanol. Dann kann die zu chromatographierende Substanz zunehmend vom Zustand der Adsorption am Festkörper in den der Lösung in dieser Oberflächenschichte übergehen, ähnlich dem Zustand wie er bei der Verteilungschromatographie ausgehend von zwei flüssigen Phasen sich ausbildet. Es gibt sich so der eingangs erwähnte Grenzfall zwischen Verteilungs- und Adsorptionschromatographie.

3. Der Vorgang, welcher dem Aufgeben der flüssigen Probe auf die Chromatographie-Säule (-Folie, -Faser) folgt und die bessere Trennung in Zonen zum Ziel hat, wird in der organischen Chromatographie "Entwickeln" genannt. In der anorganischen Chromatographie wird hierfür häufig "Auseinanderwaschen" gebraucht. Dieser Ausdruck soll auch im folgenden Bericht verwendet werden.

4. In der anorganischen Chromatographie ist in der Regel ein weiterer Vorgang nötig, nämlich Umsetzung mit einer anderen Lösung, um die einzelnen Zonen deutlich sichtbar zu machen. Hierfür ist die Bezeichnung "Entwickeln" gebräuchlich. Um Missverständnisse zu vermeiden soll hier der Ausdruck "Anfärben" benützt werden.

5. Während in der organischen Chromatographie das "Eluieren", worunter das Verdrängen des Adsorbates durch das Lösungsmittel verstanden wird, eine grosse Rolle spielt, ist dieser Vorgang in der anorganischen Chromatographie nicht immer in analoger Weise durchführbar. Durch Nachwaschen mit Wasser kann z.B. eine Hydrolyse bewirkt werden, andererseits ist ein Verdrängen durch andere gelöste Ionen, insbesonders H^+-Ion möglich. Jedenfalls ist der Mechanismus keineswegs immer eindeutig klar. "Eluieren" bedeutet demnach im Folgenden ganz allgemein ein Waschen mit Wasser, dem Lösungsmittel oder irgend einer Reagenzlösung, ohne Rücksicht darauf ob die dadurch bewirkte Verschiebung der Adsorbatzone auf blosse "Verdrängung" oder eine chemische Reaktion zurückzuführen ist.

6. Besonders wichtig ist eine Klärung des viel verwendeten Begriffes "Austausch", besonders "Ionenaustausch". Hier erscheinen folgende Begriffsbildungen zweckmässig:

Als *formaler Ionenaustausch* kann jede Reaktion zwischen einem festen, aus Ionen aufgebauten Stoff und Ionen der umgebenden Lösung nach Gleichung (1)

$$AB_{\text{fest}} + C^- = AC_{\text{fest}} + B^- \qquad (1)$$

oder analoger Kationenaustausch gelten.

Diese formale Bedingung wird jedoch auf verschiedene Weise erfüllt:

(a) durch einen *strukturkonstanten* Austausch wie er bei den hochpolymeren und

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daher unlöslichen Zeolithen und Kunstharzen vor sich geht, bei ersteren mit Kationen, bei letzteren je nach Aufbau mit Kationen oder Anionen. Hierher kann man auch die Umsetzung einer Ionenlösung mit schwerlöslichen Substanzen zählen, soferne sie keine Umwandlung der Gitterstruktur verursacht wie beispielsweise

$$Ca_5(PO_4)_3OH + F^- = Ca_5(PO_4)_3F + OH^-$$

Man kann solche Vorgänge auch als idealen Ionenaustausch bezeichnen, weil bei ihnen ein unmittelbarer Platzwechsel der Ionen an fixierten Gitterplätzen vorliegt.

(b) Ein der allgemeinen formalen Austauschgleichung ebenfalls entsprechender Vorgang, welcher jedoch die Struktur des Adsorbens verändert, also *strukturvariant* ist, liegt beispielsweise vor, wenn das kubische Silberchlorid durch Reaktion mit Br^--Ion aus der umgebenden Lösung in das hexagonale AgBr übergeführt wird, auch wenn sich die Umsetzung nur auf die Oberfläche beschränkt.

Für das behandelte Gebiet von speziellem Interesse ist die Umsetzung von Aluminiumoxyd mit wässriger Metallsalzlösung z.B. nach Gleichung (2)

$$Al(OH)_3 + MeX_2 = Al(OH)_2X + Me(OH)X \qquad (2)$$

welcher an der Oberfläche des Adsorbens zur Bildung zweier fester Phasen führen kann. Die formale Austauschreaktion stellt sich analytisch als eine Fixierung der gesamten Verbindung MeX_2 auf dem Adsorbens dar. Solche Vorgänge wurden auch vielfach als unter dem Sammelbegriff "äquivalente Adsorption" (von Kation und Anion) beschrieben. Sie sollten für diesen Fall sinnvoller bis zur Grundreaktion der Fällung zurück verfolgt werden, da sie ja im wesentlichen über die Lösung gehen.

Sofern das eine Reaktionsprodukt löslich ist, stellt sich die Reaktion auch analytisch als Austauschreaktion von Kation und Anion dar. Dabei kann entweder das Aluminium in Lösung gehen, z.B. als basisches Chlorid oder das aufgegebene Metallion, z.B. Tl^+ als Hydroxyd, welches erst tiefer unten auf der Säule adsorbiert wird und eluierbar ist, während das zugehörige Anion, insbesonders wenn es Sulfat ist, wesentlicher fester am Al_2O_3 fixiert bleibt. Im ersteren Falle entsteht eine Fällung des basischen Metallchlorides auf der Säule, im letzteren handelt es sich um einen strukturvarianten, also nur formalen Austausch der Anionen.

Für Fällungsreaktionen dieser Art spielt die Oberfläche des Adsorbens sicherlich auch eine wesentliche Rolle wie bei allen "topochemischen Vorgängen".

B. ENTWICKLUNG UND GLIEDERUNG

Zur Frage der Entwicklung der anorganischen Chromatographie im Allgemeinen und des speziellen Gebietes seien einige chronologische Bemerkungen vorangestellt. Die erste Form der Chromatographie anorganischer Salzlösungen ist sicherlich das "Filterverfahren", welches zur Wasserenthärtung mit natürlichen Zeolithen seit gerade 100 Jahren von Eichhorn[20] und später mit den von Gans[42] entwickelten synthetischen Nachbildungen, den Permutiten, durchgeführt wird. Papierchromatographische Einzelversuche liegen ebenfalls schon lange zurück, da bereits SCHÖNBEIN[153] das verschieden weite Zurückbleiben von Salzen gegenüber der Wasserfront im Papierstreifen beobachtet hat.

Eindeutig eine Fällungschromatographie und zwar auf imprägniertem Papier stellt erst die Tüpfelanalyse dar, insbesonders wenn zwei Ionen nebeneinander nachgewiesen werden. Der erste Fall dieser Art wurde 1930 von FEIGL[27] beschrieben. Die Tüpfelanalyse hat sich bekanntlich zu einem selbständigen Arbeitsgebiet entwickelt[26], welches hier nicht weiter behandelt werden kann.

Die Problematik der Vorgänge bei der chromatographischen Trennung von anorganischen Ionen begann erst mit den Versuchen von SCHWAB[154] an Aluminiumoxyd, welches auf Grund seiner ausgezeichneten Eigenschaften in der organischen Chromatographie auf die der anorganischen Verbindungen übertragen wurde. Die Deutung des Mechanismus erwies sich wegen der Überlagerung verschiedener Vorgänge hier jedoch als besonders schwierig und die Ergebnisse längst nicht so günstig wie in der organischen Chemie.

SCHWAB selbst hat mehrere Begründungen für den Verlauf der Vorgänge gegeben bezw. diskutiert, nämlich:

1. Permutoide Verdrängung von Na^+ durch die chromatographierten Kationen, d.h. Austauschadsorption[155].

2. Analoge Austauschadsorption für Anionen[157].

3. Bildung basischer Doppelaluminate[161–163, 165]. Er lehnt ab die Fällung von Hydroxyden[161], von basischen Salzen[165] und Austausch des Metalls gegen Al^{3+}[155].

In den zahlreichen Publikationen der folgenden 20 Jahre wurden "Austausch", "Adsorption" und "Fällung" in verschiedenen Variationen und sich oft widersprechend als Ursache der Fixierung anorganischer Ionen besonders an Al_2O_3-Säulen angesehen. Auch in der neuesten zusammenfassenden Buch- und Zeitschriftliteratur[8, 76, 141, 179, 180, 209] ist daher eine klare Aufteilung der anorganischen Chromatographie in diese Gruppen nicht zu finden. Im folgenden Bericht nimmt die Diskussion ihrer Ursachen einen relativ breiten Raum ein, gegenüber der Auswertung für qualitative und quantitative Zwecke, welche wohl nicht ganz den seinerzeit darein gesetzten Erwartungen entspricht.

Da, wie erwähnt, die meisten Versuche an Al_2O_3 gemacht wurden, werden zunächst diese nach den drei wichtigsten Erklärungsversuchen: Austausch, (physikalische) Adsorption und (hydrolytische) Fällung besprochen, ferner das Verhalten der Anionen, das auch für die Kationentrennung wichtig ist. Eine weitere Gruppe bilden die Versuche mit Imprägnierungen auf verschiedenen Trägern, welche den Übergang zu festen Adsorbentien, welche selbst mit den Salzlösungen reagieren, bilden. Die Verwendung für quantitative Zwecke, insbesonders im Mikromasstab ist bedeutend, eine weitere Anwendung ergibt sich für die Reinigung von Salzlösungen. Spezielle Methoden ergeben sich aus der Anwendung von Komplexbildern und radioaktiven Indikatoren und für verschiedene Elementgruppen.

Innerhalb der folgenden Abschnitte sind die Arbeiten im wesentlichen nach chronologischer Folge besprochen.

C. SPEZIELLER TEIL

1. *Chromatographie von Kationen an Al_2O_3 unter dem Gesichtspunkt des Ionenaustausches*

In den ersten Publikationen erklärt Schwab[154,155] die Fixierung der Kationen im wesentlichen durch Austausch des Schwermetallions gegen Na^+, welches im Filtrat nachgewiesen werden kann. Die meisten Al_2O_3-Präparate für chromatographische Zwecke enthalten aus dem Herstellungsprozess Natrium als Carbonat oder Aluminat. Es kann nach Schwab durch das Metall ersetzt werden nach Gleichung (3):

$$>\mathrm{Al{-}O{-}Na} + \mathrm{Me^{n+}}/\mathrm{n} = >\mathrm{Al{-}O{-}Me}/_{\mathrm{n}} + \mathrm{Na^+} \qquad (3)$$

wodurch es zur Bildung von spinellartigen Aggregaten kommen soll. Durch paarweise Versuche ergibt sich für Kationen, welche aus komplexfreier Lösung chromatographiert werden können, folgende Reihung:

$$\mathrm{H^+},\ \begin{matrix}\mathrm{Cr^{3+}}\\ \mathrm{Fe^{3+}}\\ \mathrm{Hg^{2+}}\end{matrix},\ \mathrm{UO_2^{2+}},\ \mathrm{Pb^{2+}},\ \mathrm{Al^{3+}},\ \mathrm{Cu^{2+}},\ \mathrm{Ag^+},\ \mathrm{Zn^{2+}},\ \begin{matrix}\mathrm{Co^{2+}}\\ \mathrm{Ni^{2+}}\\ \mathrm{Cd^{2+}}\\ \mathrm{Fe^{2+}}\end{matrix},\ \mathrm{Tl^+},\ \mathrm{Mn^{2+}}$$

Flood[34] nimmt für seine Versuche mit auf Papier niedergeschlagenem Al_2O_3 dieselbe Austauschadsorption an und auch Venturello[204a] hält diesen Vorgang für eine mögliche Erklärung. Lindner[92-97] spricht bei seinen Versuchen an Aluminiumoxyd mit radioaktiven Indikatoren ebenfalls von Austauschadsorption. Auch Hesse[68] formuliert in analoger Weise den Ersatz von Na^+ durch Ag^+. Er weist auch auf die Tatsache hin, dass Säuren (Anionen) von Aluminiumoxyd besser aus saurem, Basen (Kationen) aus basischem Milieu adsorbiert werden, was nach seiner Ansicht am besten durch Ionenaustausch erklärt werden kann.

Jacobs[73] führt die schon von Schwab[154] erwähnte Tatsache, dass die Kationenzonen unmittelbar aneinander schliessen auf den komplizierenden Einfluss der gleichzeitigen Anionenadsorption zurück und erklärt die Adsorption durch Ionenaustausch und molekulare Fixierung. Er weist aber in dem Zusammenhang darauf hin, dass die Kationen mit Ausnahme von H^+ unfähig sind, in grösserem Ausmass andere adsorbierte Kationen zu verdrängen.

In ähnlicher Weise erklärt Gapon[43,45] die Fixierung der Kationen an Al_2O_3 durch Austausch, sofern nicht gleichzeitig Anionen festgehalten werden. Robinson[142] lässt nur Ionenaustausch für Kationen gelten im Gegensatz zur (physikalischen) Adsorption der Molekel.

Fischer[29] stellt durch statische Versuche, aus welchen er auf die chromatographischen Vorgänge rückschliesst fest, dass ein Austausch von Na^+ gegen das Kation in äquivalenten Mengen stattfindet, wobei offen bleibt, ob es sich um Fällung oder permutoiden Austausch handelt. Daneben findet eine äquivalente Adsorption von Kation und Anion des Salzes statt. Umland und Fischer entwickeln später[194,195] die Vorstellung, dass es sich bei dem primären Vorgang der Adsorption ionisierter Salze aus wässriger Lösung um einen gekoppelten Austausch von Kationen einerseits und Anionen anderseits handelt unter Mitwirkung von H^+ und OH^-, eine Vorstellung, die sich schon bei SACCONI[145, 149] findet. Ausführlich werden die aus statischen Versuchen von UMLAND[197–199] erhaltenen Ergebnisse diskutiert und zu einer formalen Ionenaustauschtheorie für die Adsorption von Elektrolyten entwickelt.

D'ANS[16, 17] sieht ebenfalls nicht nur Na^+, sondern auch H^+ als austauschbar gegen das Metallion an, wobei er sich zum Teil auch der Suspensionsversuche als Grundlage seiner Überlegungen bedient. HAYEK[66] weist darauf hin, dass statische Versuche nur mit Vorbehalt Rückschlüsse auf die Verhältnisse an der Säule gestatten, weil sich bei letzteren nicht die den statischen Gleichgewichten entsprechenden pH-Werte einstellen, sondern viel extremere messbar sind. Ferner wird in Zusammenfassung verschiedener bekannter Tatsachen darauf hingewiesen, dass der Natriumgehalt des Aluminiumoxydes, welcher nach Gl. (3) formal einen Ionenaustausch bewirkt, durch Umsetzung in folgenden Stufen dargestellt werden kann:

$$\rangle Al{-}O{-}Na + H_2O = \rangle Al{-}OH + NaOH \tag{4}$$

$$NaOH + MeX_2 = Me(OH)X + NaX \tag{5}$$

Die Hydrolyse des Natriumaluminates nach Gl. (4) ergibt sich aus der Messung des pH-Wertes einer Al_2O_3-Suspension nach HESSE[69] für die üblichen Präparate durch ihren Anstieg auf 9–10. Unter diesen Bedingungen wird auch auf einer Säule kein Austausch von Schwermetallionen möglich, sondern Fällung basischer Salze oder Hydroxyde.

Um die Komplikation durch den Natriumgehalt des Al_2O_3 zu umgehen sind schon frühzeitig Versuche mit *reinem Aluminiumoxyd*, durch Fällung mit NH_3 oder aus Amalgam hergestellt, durchgeführt worden. Es ergab sich bereits bei SCHWAB[155] mit einem solchen Präparat zwar die gleiche Reihung der Kationen, jedoch eine grössere Zonenlänge und eine gewisse Eluierbarkeit. VENTURELLO[205] untersuchte auch das Reflexionsspektrum von adsorbiertem $CuSO_4$ und stellte fest, dass es sich wenig von dem des reinen Salzes unterschied. Hieraus schliesst er, dass keine chemische Reaktion vorliegt. SCHWAB[162] hatte hingegen von Na-haltigem Adsorbens Spektren zwischen denen des Ions und des Hydroxydes erhalten, ähnlich dem des entsprechenden basischen Salzes. Er schliesst daraus jedoch, dass der Bindungszustand nicht mit Aluminaten, sondern eher mit basischen Doppelsalzen, wie etwa "basischen Aluminatnitraten oder -Sulfaten" vergleichbar sei. Dies kann man so deuten, dass das Natrium durch das Schwermetallion ersetzt wird, welches noch eine OH-Gruppe und ein Äquivalent Anion, etwa Nitrat gebunden enthält.

FRICKE[40], SACCONI[149] und UMLAND[195] stellen fest, dass Aluminium durch das Metallsalz in Lösung geschickt wird, was einen formalen Austausch bedeutet. Versuche von GRASSHOF[57] an alkalifreiem Al_2O_3, welche mit den andern Versuchen an reinem Oxyd nicht übereinstimmen, finden später[58] ihre Erklärung durch den Calciumgehalt des Präparates.

FRICKE[39] vertritt ausdrücklich die Meinung, dass die Fixierung von Fe^{3+}, Cu^{2+} und Co^{2+} auf reinem Al_2O_3 durch den Austausch der Salzanionen gegen die OH-

Gruppen des Adsorbens zu Stande kommt, wobei die Reihung der zunehmenden Basenstärke der Schwermetallbasen entspricht.

Nach FISCHER[29] wird auf Na-freier Tonerde (im statischen Versuch) Cu^{2+} und Cl^- in völlig äquivalenten Mengen festgehalten, im Gegensatz zum Na-hältigen Präparat wo das Kation Cu^{2+} im Überschuss bleibt. Ersterer Vorgang entspricht Gl. (2), nach FISCHER ist das Adsorbat zum Teil eluierbar, dies kann dadurch erklärt werden (HAYEK[66]) dass es sich um ein reversibles Gleichgewicht handelt, entsprechend Gl. (2): $Al(OH)_3 + MeX_2 = Al(OH)_2X + Me(OH)X$, welches bei grösserer Verdünnung sich von rechts nach links verschiebt. Tatsächlich ist jedes basische Salz nur unter einer gewissen Mindestkonzentration des Neutralsalzes stabil, wenn diese unterschritten wird, hydrolysiert das basische Salz, das führt zur Bildung von MeX_2 einerseits und $Me(OH)_2$ andererseits, welches mit $Al(OH)_2X$ weiter zu $Me(OH)X$ reagieren muss, sodass MeX_2 zum grössten Teil rückgebildet werden kann. Wenn das Gleichgewicht aus irgend welchen Gründen nach rechts verschoben wird, so bildet sich in grösseren Mengen das basische Salz wie SCHÄFER[151] röntgenographisch bei $Cu_2(OH)_3Cl$ festgestellt hat.

2. *Chromatographie von Kationen an Al_2O_3 unter dem Gesichtspunkt der (physikalischen) Adsorption von Ionen und Molekeln*

SCHWAB[155] stellte fest, dass $HgCl_2$ sich an der Al_2O_3-Säule grundsätzlich anders verhält, nämlich schon beim Auseinanderwaschen rasch verschwindet oder viel tiefer festgehalten wird, als die anderen Hg- und anderen Schwermetallsalze. Er zieht jedoch hieraus keine weitergehenden Folgerungen.

VENTURELLO[201–206] führt die chromatographische Fixierung von allen Kationen, zumindest in erster Stufe, auf die Bildung einer elektrischen Doppelschichte zurück, die Festigkeit der Bindung sei abhängig von Ladung, Grösse und Polarisierbarkeit der Ionen und nur in besonderen Fällen sollen "spezifische Affinitäten" diese Kräfte überwiegen. Ein gradueller Verlust der Hydratwassermolekel mit steigender Temperatur erhöht die Bindungsfestigkeit und ihren irreversiblen Charakter.

JACOBS[73] begründet die Fixierung von Kationen, soweit sie gleichzeitig mit Anionen vor sich geht, durch molekulare Adsorption. Die Grösse dieser äquivalenten Adsorption von Kationen und Anionen geht parallel mit dem kovalenten Charakter des Salzes.

SHIBATA[176] bestätigt die von SCHWAB gefundene Kationenreihung, erklärt sie aber als abhängig vom Radius des hydratisierten Ions und seiner Ladung.

FISCHER[29, 30, 32] führt den Teil der Kationenadsorption welcher mit äquivalenten Beträgen Anion und eluierbar stattfindet auf elektrostatische Kräfte zurück, die entsprechenden Versuche sind allerdings im wesentlichen nicht auf einer Säule, sondern statisch durchgeführt. FISCHER, SCHÄFER und NEUGEBAUER[41, 120, 150] stellen physikalische Adsorption (= äquivalente Adsorption) besonders an einer mit Säure formierten Al_2O_3-Säule fest, wobei sie offen lassen ob Molekel, Einzelionen oder Ionenpaare adsorbiert werden. Wesentlich ist für sie die Eluierbarkeit der Ionen.

NEUGEBAUER UND SCHÄFER[120] berichten über die erfolgreiche Trennung der Alkalien an alkalifreiem Al_2O_3 (Präparat Woelm, welches Ca enthält), wobei mit reinem Wasser eluiert und durch Leitfähigkeitsmessung die Mengenbestimmung durchgeführt wird. An der sauer formierten Säule werden auch Mn, Ni, Cd und Hg paarweise voneinander getrennt. Die Zonen erscheinen im Gegensatz zu denen an Na-hältigen Säulen durch unbelegte Zwischenräume getrennt.

ERLENMEYER[23] diskutiert die Möglichkeit, dass bei den Versuchen SCHWABS mit Kationen in einigen Fällen die O-Atome des Al_2O_3 sich unter Bildung der H-Brücken beteiligen.

3. *Chromatographie von Kationen an Al_2O_3 unter dem Gesichtspunkt der Hydrolyse und Fällung*

In den ersten Arbeiten SCHWABS wird der Austausch von Na^+ gegen das Metall betont und andere Adsorbentien wie ZnO und MgO werden als zu stark fällend und daher ungeeignet bezeichnet[155]. Derselbe Autor[161] lehnt jedoch die Möglichkeit der Hydroxydfällung ab mit der Begründung, dass die Reihung an der Säule nicht der der Löslichkeiten entspricht. Auch die Bildung basischer Salze wird abgelehnt, weil nach HAYEK[65a] ihre Bildung stark anionabhängig sei, was bei den chromatographischen Versuchen nicht bemerkt wird. Dieser Autor macht jedoch darauf aufmerksam[66], dass diese Ablehnung nicht stichhaltig ist, weil die Beeinflussung der Kationen durch das gleiche Anion in die gleiche Richtung geht, d.h. z.B. dass alle basischen Phosphate gegen Hydrolyse stabiler sind als die Sulfate, und diese wieder als die Nitrate, sodass ein Anionenzusatz alle vorhandenen Kationen in annähernd gleicher Weise beeinflusst.

Auf Grund der Absorptionsspektren des Adsorbates, welche zwischen denen der Salzlösungen und denen der Hydroxyde liegen, kommt SCHWAB später[162] zu dem Schluss, dass es sich um die Bildung basischer Doppelsalze handelt, er nennt beispielsweise basische Aluminatnitrate und -sulfate. Diese Annahme erscheint bei wörtlicher Auslegung gezwungen, da unter den herrschenden pH-Verhältnissen (zwischen 5 und 9) Aluminate nicht beständig sind. Sie wird verständlicher, wenn man statt basischer Aluminate basische Aluminiumsalze setzt. Jedenfalls wird dieser Annahme gegenüber einer von SIEWERT[177] vertretenen Ansicht, dass es sich um Fällung von Carbonaten handelt, später von den Autoren gemeinsam[163] der Vorzug gegeben. SIEWERT allein vertritt allerdings die Meinung[178], dass die Fixierung der Kationen durch Austausch von OH-Gruppen des Al_2O_3 gegen die Anionen des Salzes und eine so bedingte Bildung von basischen Salzen bezw. Hydroxyden zu stande kommt, also eine Fällung darstellt.

JACOBS[73] lehnt eine hydrolytische Adsorption nach Gl. (6)

$$Me^{++} + H_2O = MeOH^+ + H^+ \tag{6}$$

ab, mit der Begründung, dass eine Erhöhung der Acidität der Lösung durch die Adsorption nicht feststellbar sei. Dies, obwohl dem Autor bekannt erscheint, dass das H^+-Ion gut befähigt ist andere Kationen von der Säule zu verdrängen, also auf diese Weise inaktiv gemacht werden kann.

Kubli[87] beschäftigt sich ebenfalls mit der Trennung der Kationen und sieht sie als Fällung von Metallcarbonaten oder Hydroxyden an, im Gegensatz zu den Austauschvorgängen, welche die Chromatographie der Anionen beherrschen.

Den Ausdruck hydrolytische Adsorption verwendet auch Fricke[39] für die Umsetzung zwischen KCl und $Al(OH)_3$, welche eine Steigerung des pH-Wertes auf über 9 durch formalen Ionenaustausch verursacht, nach Gl. (7)

$$Al(OH)_3 + KCl = Al(OH)_2Cl + KOH \tag{7}$$

Sacconi[145–149] stellt fest, dass die hydrolytische Adsorption von Kationen durch die Pufferwirkung des Al_2O_3 begünstigt wird. Der Hydrolysengrad der Salzlösungen wird als parallel mit der Reihung der Kationen an Al_2O_3 erkannt[149]. Die Ionenhydrathülle ist ein essentieller Faktor bei der Adsorption (vergl. Venturello[201–206]), die polarisierten Wassermolekel werden successive durch OH^- ersetzt nach Gl. (8)

$$6[M(H_2O)_6]^{2+} + Al_2O_3 + 9H_2O = 6[M(H_2O)_5OH]^+ + 2[Al(H_2O)_6]^{3+} \tag{8}$$

Das entstandene Ion $[Al(H_2O)_6]^{3+}$ wird in der Folge wieder hydrolysiert, z.B. zu $[Al(OH)_2]^+ + H^+$. Hierzu wird bemerkt[66], dass bei den herrschenden pH-Verhältnissen von Anfang an nur Hydroxykationen des Aluminiums stabil sind und ein $[Al(H_2O)_6]^{3+}$ sich dort nicht bilden kann.

Fischer[30] stellt fest, dass die Reihung der seltenen Erden an Al_2O_3 der Hydroxydlöslichkeit parallel geht, zieht aber daraus keine Schlüsse auf den Mechanismus.

Ausführlich hat sich Schäfer[150, 151] mit dem Problem der Fällung auseinandergesetzt und kommt zu dem Schluss, dass z.B. $CuCl_2$ an reinem Al_2O_3 neben der physikalischen (äquivalenten) Adsorption als basisches Chlorid gefällt wird nach Gl. (9)

$$Al_2O_3 + 3H_2O + 4CuCl_2 = 2Cu_2(OH)_3Cl + 2AlCl_3 \tag{9}$$

(Hierzu gilt wie oben, dass $AlCl_3$ neben basischem Kupfersalz nicht stabil ist, sondern ein basisches Ion wie $Al(OH)_2^+$ bildet[66]). Al_2O_3 wirkt als schwache Festbase in ähnlicher Weise wie andere, stärkere Festbasen: MgO, Anionenharzaustauscher Wofatit M, usw.

Umland[195] hält eine Fällung wie Schäfer sie beschreibt für möglich, wenn bei dem von ihm geforderten Austausch von Anion und Kation der Anionenaustausch erheblich rascher ist und so OH^--Ionen im Überschuss liefert.

Die Feststellung der Zunahme der Belegungsdichte am Al_2O_3, gleichsinnig mit der Temperatur, hat als erster Venturello[205], dann umfassender Heinrich[67] gemacht, sie spricht für den Vorgang der Hydrolyse und gegen der physikalischen Adsorption.

Yasunaga[210] hält aus dem besonderen Verhalten von $HgCl_2$ und aus pH-Messungen den Fällungsvorgang für die allgemeine Ursache der chromatographischen Fixierung der Kationen. Specker[181] stellt fest, dass die Oberflächenaktivität des Al_2O_3 parallel seiner Löslichkeit in Fluoridlösungen geht, woraus sich die Bedeutung der OH-Gruppen in der Oberfläche für den Adsorptionsvorgang ergibt.

Hayek[64, 66] schliesst aus der gleichen Reihung der Kationen an Al_2O_3, Glaspulver und anderen, den pH-Wert erhöhenden Adsorbentien, dass die Ursache eine pH-bedingte Fällung ist.

Literatur S. 349/353.

D'ANS[17] gibt der Meinung Ausdruck, dass an Al_2O_3, aus welchem das Na^+ nicht ausgewaschen wurde, zusätzlich zum Ionenaustausch eine Fällung basischer Salze eintreten kann. D'ANS[18] richtet sich jedoch gegen die Ansicht der Fällung, weil entsprechend der Phasenregel ihre Existenz unmöglich erscheint. Demgegenüber weist HAYEK[66] darauf hin, dass die Anwendung der Phasenregel auf das nicht im statischen Gleichgewicht befindliche Säulensystem nicht statthaft sei. In der gleichen Arbeit werden verschiedene experimentelle Tatsachen aus der verstreuten Literatur und weitere eigene Versuche diskutiert, welche für das Auftreten von Fällungen am Al_2O_3 sprechen, insbesonders auch die Bildung extremerer pH-Werte als sie bei der statischen Prüfung der Adsorbentien nach HESSE[69] zu messen sind, auf der Säule. Ferner wird auf die zu wenig beachtete Tatsache hingewiesen, dass basische Salze von Schwermetallen nur im sauren pH-Gebiet stabil sind und niederer pH-Wert daher kein Argument gegen die Bildung solcher Fällungen sein kann.

NODDACK[121] diskutiert ausführlich die Anschauungen über die Ursachen der Fixierung auf der Al_2O_3-Säule und entscheidet sich für hydrolytische Adsorption und Bildung basischer Salze. Es wird eine weitgehende Parallelität des Hydrolysengrades der Salzlösungen mit ihrer Reihung auf der Säule, ähnlich SACCONI[149] festgestellt. Die Bedeutung des Alkalizusatzes für eine Verbesserung der Fällung infolge Erhöhung des pH-Wertes wird betont. Auch wird eine Erweiterung der Kationenreihung gegeben.

4. *Verwendung von Al_2O_3 auf Trägersubstanzen*

Die ersten Versuche dieser Art, nämlich mit auf Papier niedergeschlagenem $Al(OH)_3$ machte, wie erwähnt FLOOD[34–38]. GOTO[56] verwendet dasselbe Material, färbt die adsorbierten Kationen mit Oxin an und erhöht die Nachweisempfindlichkeit durch UV-Fluorescenz. Dasselbe Adsorbens verwendet IIJIMA[72] für halbquantitative Versuche und auch HOPF[71]. Beide Autoren benützen auch organische Reagenzien zur Anfärbung, ähnlich auch OKA[124–127] und MURATA[109–114], welcher auch quantitative Ergebnisse erhält (s.u.). VANYARKO[200] zeigt zahlreiche Trennungen von Metallpaaren, ebenso ZOLOTAVIN[211].

BEZUGLYI[6] spricht die auf mit $Al(OH)_3$ imprägniertem Papier erhaltenen Ergebnisse als Fällungschromatographie an.

5. *Einfluss der Anionen auf die Kationadsorption an Al_2O_3*

SCHWAB[157] stellte fest, dass die Reihenfolge der Kationen unabhängig vom Anion ist. Die Art des Anions verursacht unter Umständen eine Änderung der Zonenlänge, insbesonders verkürzt Sulfat stark gegenüber Nitrat und Chlorid. Zusatz von allen Alkalisalzen kann aber auch Zonenkürzung verursachen. Dies kann verschiedene Ursachen haben: NODDACK[121] und ebenso UMLAND[195] verweisen auf die Erhöhung der Alkalität nach Gl. (7) (s.o.), HAYEK[63] auf die Beeinflussung der Löslichkeit basischer Salze durch die Erhöhung der Anionenkonzentration.

JACOBS[73] findet, dass bei gleichzeitiger Anwesenheit von Cu^{2+}, NO_3^- und SO_4^{2-}

in der aufgegebenen Lösung das Cu^{2+} in einer höheren SO_4^{2-}-hältigen und einer darunter anschliessenden NO_3^--hältigen Zone festgehalten wird. Mehrfach wurde festgestellt (Gapon[49], Umland[197]), dass Kationen, die als Sulfate auf die Säule kamen, schwerer eluierbar sind als solche die als Chlorid- oder Nitratlösungen verwendet wurden. Gapon[47] fand, dass die Menge Anion, welche mit dem Kation zusammen gebunden wird, mit der chromatographischen Reihung der Anionen im Zusammenhang steht.

Fricke[39] betont, dass der Austausch des Anions gegen OH^- des Al_2O_3 wesentlich für die Kationenfixierung ist, in ähnlicher Weise erklärt Siewert[178] die Bindung des Kations durch Fällung basischer Salze. Auch die Anschauungen von Umland und Fischer[194, 195] über den gekoppelten Ionenaustausch (vgl. unter 1, S. 338) sprechen für die Bedeutung des Anioneneinflusses bei der Fixierung der Kationen.

Hayek[63] wies nach, dass Anionen, welche schwerer lösliche Verbindungen bilden als die entsprechenden Hydroxylverbindungen, sehr wohl geeignet sind die Reihung der Kationen zu beeinflussen. So wird Ag^+ durch PO_4^{3-} höher oben auf der Al_2O_3-Säule fixiert und einen noch umfassenderen Einfluss haben Sulfid- und $[Fe[CN)_6]^{4-}$-Ion auf die Kationenreihung (vgl. 7, S. 345, 346).

6. *Anionenadsorption an Al_2O_3 und anderen Metalloxyden*

Bereits Schwab[157] hat festgestellt, dass eine Al_2O_3-Säule besonders gut befähigt ist, Anionen in chromatographischer Reihung festzuhalten, wenn sie einer Behandlung mit Säure (saurer Formierung) unterzogen wurde. Er stellt folgende Reihung der Anionen fest:

$$OH^-,\ PO_4^{3-},\ F^-,\ \begin{matrix} Fe(CN)_6^{4-} \\ CrO_4^{2-} \end{matrix},\ SO_4^{2-},\ \begin{matrix} Fe(CN)_6^{3-} \\ Cr_2O_7^{2-} \end{matrix},\ Cl^-,\ NO_3^-,\ MnO_4^-$$

Die bevorzugte Stellung am Beginn der Reihe, wie in der Kationenreihe das H^+, nimmt hier das OH^- ein, entsprechend der puffernden Wirkung des Al_2O_3 für beide Ionen. Durch Behandlung mit den starken Säuren der am Ende der Reihe stehenden Anionen ClO_4^- und NO_3^- wird die Säule für die Adsorption der darüber stehenden Anionen geeignet. Der Vorgang wird als Austauschadsorption bezeichnet. Flood[34] bestätigt die Reihung und den Mechanismus an mit $Al(OH)_3$ imprägniertem und mit Säure formiertem Papier.

Auch Kubli[87] erkennt den Anionenaustausch an und spricht von "Tonerdepermutit", im Gegensatz zur Fällungschromatographie der Kationen. Er hält aber fest, dass die Reihenfolge der Anionen auf der Al_2O_3-Säule der Löslichkeitsfolge der basischen Aluminiumsalze entspricht. Im Sinne der Ausführungen auf S. 335 handelt es sich also um einen formalen Ionenaustausch.

Kühn[88] gibt eine Ergänzung der Reihe, genauere Untersuchungen hierüber stammen von Gapon[47] und Murata[109–114], letztere auf imprägniertem Papier. D'Ans[17] stellt fest, dass auf einer sauren Säule aus einer $CuSO_4$-Lösung SO_4^{2-} und Cu^{2+}, aus einer $CuCl_2$-Lösung nur Cl^- adsorbiert wird. Er führt dies auf die Zweiwertigkeit von Kation und Anion zurück.

MURATA[114] untersucht bei Al_2O_3 auf Papier den Einfluss des pH-Wertes auf die Zonenbreite der Polyanionen bildenden Säuren, welche stark variiert.

HAYEK[65] vergleicht die Anionenreihung an Al_2O_3 mit der an ZnO, PbO, La_2O_3 und Bi_2O_3 und findet gänzlich verschiedene Anordnungen, welche weitgehend mit der Schwerlöslichkeit der entsprechenden Metallsalze parallel gehen. Hieraus schliesst er auf Oberflächenreaktionen in Form von Fällungen, welche formal als Austausch erscheinen. Er stellt im Vergleich von Suspensions- und Säulenversuch sauer formierter Oxyde fest, dass der Säulenversuch extremere pH-Werte zeigt, sodass Suspensionsversuche nicht zuverlässige Aussagen für die Verhältnisse bei der Säulenchromatographie gestatten.

7. *Andere anorganische Adsorbentien*

SCHWAB[155] hat verschiedene Oxyde, $BaSO_4$ und Glasstaub als zu wenig aktiv für die Kationenchromatographie, ZnO, MgO, als zu stark basisch, Floridin, Kieselgel und $Ca_3(PO_4)_2$ als brauchbar bezeichnet, ohne jedoch Angaben über die Ionenreihung an diesen Substanzen zu machen. FLOOD[34] erhält bei Verwendung von Papier, welches mit $Cr(OH)_3$ imprägniert ist, zumindest gleich gute Erfolge wie mit Al_2O_3.

BACH[2] erhielt Trennung von Cu^{2+} und Cd^{2+} und anderen Kationen auf ZnS als Adsorbens, hingegen gab $Zn_2[Fe(CN)_6]$ keine guten Resultate. Auch KUTZELNIGG[89] trennt verschiedene, insbesonders edle Metalle an einer ZnS-Säule und bezeichnet seine Methode als "Filterverfahren".

MILICEVIC[102] verwendet eine $PbCl_2$-Säule zur Trennung von Br^- und J^- sowie eine Zn-Pulversäule zur Trennung von Ag^+, Cu^{2+}, und Pb^{2+} durch Reduktion in Zonen und nennt seine Methode "Chemigraphie". KOMLEV[80] verwendet eine Ag_2SO_4-Schichte auf einem Träger für Halogenidtrennungen, ebenso TEIGE[190].

SEN[166–170] führte verschiedene Kationentrennungen an Stiften von Schreibkreide durch, welche aus $CaSO_4$ und $CaCO_3$ bestehend wohl im wesentlichen hydrolytisch wirken.

GAPON[43] führt Trennungen verschiedener Kationen an Bariumaluminat und ZnO auf Austausch zurück. Hingegen spricht er von Fällungschromatographie bei Verwendung von Säulen verschiedener Träger (Al_2O_3, SiO_2), welche mit verschiedenen Fällungsreagentien imprägniert sind, wie Na_2SiO_3, KJ, Ag_2SO_4 usw.[48, 50, 52]. CETINI[12, 13] benutzt Cellulose und anorganische Träger, welche er mit $K_2H_2Sb_2O_7$, Na_3AlO_3 und anderen Lösungen imprägniert zur Trennung zahlreicher Kationen. MILONE[103–108] trennt an basisch reagierendem SiO_2 Kationen und stellt eine Reihe auf, welche mit der von SCHWAB analog ist. Versuche von MILONE[107] in Gelatinegel, welches mit Arsenit, Borat usw. gemischt ist, sollen die nachherige mechanische Trennung der Zonen erleichtern. KOPYLOVA[81] verwendet $[Fe(CN)_6]^{4+}$ auf Al_2O_3 zur quantitativen Bestimmung von Fe neben Co und Mn.

HAYEK[63] zeigt, dass Al_2O_3 in Gegenwart von PO_4^{3-} die gleiche Kationenreihung wie ohne dieses zeigt, mit Ausnahme des Silbers, welches wegen seiner Schwerlöslichkeit als Phosphat höher oben fixiert wird, insbesonders auch auf einer Säule von Calciumphosphat, welches sonst wie Al_2O_3 reiht. Ganz andere Reihungen werden auf

Calcium-Kalium-Ferrocyanid und Zinksulfid erhalten, wo, schon aus der Farbe erkennbar, die entsprechenden schwerlöslichen Salze entstehen. Betreffend das Verhalten verschiedener Metalloxyde gegen Anionen vgl. HAYEK[65] (siehe S. 345).

UMLAND[196] benützt Kieselgel für die Kationentrennung und stellt Kationenaustausch und Adsorption unter Bildung von Oberflächenverbindungen fest, welche der Fällung von Hydroxyden und basischen Salzen ähnlich erscheint.

KRAUS[84–86] verwendet ZrO_2 und andere Metalloxyde nach Vorbehandlung mit Basen oder Säuren für die chromatographische Abtrennung von Kationen oder Anionen, $Zr_3(PO_4)_4$ von Kationen, welche er mit NH_4Cl eluieren kann. Er nimmt Austauschvorgänge als Grundlage dieser Reaktionen an, doch scheinen auch Fällungsreaktionen möglich.

8. *Organische Adsorbentien*

Als erster hat ERLENMEYER[23–25] organische Fällungsreagenzien für die Säulenchromatographie benutzt und zwar vor allem Hydroxychinolin und Violursäure auf Kieselgur oder Stärke. Er stellt eine Parallelität der Reihung mit der Löslichkeit der entsprechenden Salze fest. Violursäure eignet sich auch zur Trennung der Erdalkalien und Alkalien. ROBINSON[143, 144] arbeitet mit demselben Material, erweitert die Kationenreihe und führt quantitative Bestimmungen durch (s.u.).

Weitere organische Fällungsmittel wie Benzoinoxim prüft HOPF[71], SHEMYAKIN[172–175] verwendet Naphtochinolin und Cupferron und trennt z.T. durch Eluieren mit CNS^-. BACH[2–4] benützt Nitrosonaphtol, Dimethylglyoxim, Dithizon und andere organische Reagenzien als Säulenmaterial. BURRIEL-MARTI[9] bestimmt mit letzterem Nickel, DEAN[19] mit Nitroso-R-Salz Cobalt.

NAGAI[115–118] arbeitet mit einer Oxin-Säule, mit und ohne Lösungsmittelgemischen, hierbei fällt Eisen als $Fe(OH)_3$ zwischen den Oxinaten von Cobalt und Zink. Papier imprägniert mit anorganischen und organischen Fällungsmitteln verwendet VYAKHIREV[208] für qualitative Chromatographie und VANYARKO[200] führt zahlreiche Trennungen von Metallpaaren mit Oxin durch. Andere Versuche mit organischen Reagenzien sind im Abschnitt 9 (s.u.) angeführt.

9. *Quantitative Bestimmungen und Spurennachweis*

Von mehreren Seiten wurde festgestellt, dass die relative Zonenlänge auf Al_2O_3 kein verlässliches Mass der Mengenverhältnisse gibt und überhaupt keine vollkommenen Trennungen darauf erzielt werden können[32, 73, 87], wenn nicht besondere Voraussetzungen gegeben sind[45, 78, 100, 175, 182–184]. Allgemein scheint die Verwendung von ausgesprochenen Fällungsreagenzien bessere Trennungen zu ergeben[25, 72, 102]. Vielfach wird empfohlen, zuerst die üblichen qualitativen Gruppentrennungen durchzuführen[7, 28, 133, 158, 159, 164, 210].

Am besten eignet sich verschiedenes Säulenmaterial zur Bestimmung kleiner Mengen *einzelner* Ionenarten. Schon SCHWAB[155] kann Fe^{3+} und Cu^{2+} bis zu 1 γ nachweisen und erniedrigt später[160] die Erfassungsgrenze von Fe^{3+} auf 0.01 γ und von

Cu^{2+} auf 0.05 γ, bei Trennung von Fe und Cu im Verhältnis 1:1 auf je 0.1 γ. VENTURELLO[204] bestimmt Mikromengen von Mg auf Al_2O_3 und PINTEROVIC[139] Spuren von Fe^{3+} in reinem NaCl. LECOQ[90] prüft auf Pb^{2+} in Wasser, PFAU[138] kleinste Mengen Kupfer. BEAUCOURT[5b] weist Fe^{3+} als Berlinerblau neben der 10,000 fachen Menge Cr oder Co nach. MEINHARD[101] drückt die Erfassungsgrenze herunter durch Verwendung von Al_2O_3 auf Objektträgern. TANAKA[186-189] weist noch 0.001 γ Co und Ni auf Al_2O_3 durch Anfärbung mit $(NH_4)_2S$ oder mit Dithizon nach. BURRIEL-MARTI[9] kann 1 Teil Ni neben der 10,000-fachen Menge Co auf Dimethylglyoxim (Träger $CaCO_3$) nachweisen. ROBINSON[143, 144] verwendet Hydroxychinolin in Stärke-Säulen für die quantitative Bestimmung von Zn in Cu-Ni-Zn und Ag in Cu-Ni-Ag-Legierungen.

SHEMYAKIN[174] findet Spuren Eisen in Schwefelsäure auf SiO_2 und BACH[3] Co in Verdünnung von 10^{-9} auf Al_2O_3 mit Nitroso-β-naphtol. ASHIZAWA[1] erkennt 0.005 γ Pd mit Dithizon. KEMULA[77, 78] eluiert Adsorbate und bestimmt Co neben der 100-fachen Menge Cu polarographisch im Mikromasstab. NYDAHL[123] bestimmt Schwefel aus Stahl als Sulfat auf Al_2O_3. KHOKLOVA[79] und YASUNAGA[210] weisen Spuren von Metallen in pharmazeutischen Präparaten nach. KOMLEV[80] bestimmt kleine Mengen Halogenid an Ag_2SO_4-Säulen.

BALLCZO[5a] reichert Strontium auf einer alkalisch vorbehandelten Al_2O_3-Säule für eine Mikrobestimmung an. Er trennt Ba von Sr an einer mit HNO_3 vorbehandelten Säule in Mengen von 250 γ mit einem Fehler von ± 1%[5]. OSHCHAPOVSKI[136, 137] bestimmt kleinste Mengen Nickel neben grossem Überschuss von Cu, Co oder Fe auf einer mit Dimethylglyoxim behandelten Trägersäule. MURATA[112] wertet die Flächenbedeckung imprägnierter Papiere für die Eisenbestimmung aus und kommt auf 10–100 γ Fe herunter, durch Reflexionsspektralanalyse bis auf 1 γ. TROITSKII[193] bestimmt mehrere Metalle in der Menge von 0.5 bis 1 γ pro Liter mit Fällungsreagentien auf Papier.

10. *Chromatographische Reinigung von Salzlösungen*

Zur Reinigung von Metall- insbesonders Zinksalzlösungen für die Darstellung von Phosphoren eignet sich Al_2O_3 nach TIEDE[191] gut, SCHIKORE[152] nimmt für diesen Zweck auch mit organischen Fällungsreagentien imprägnierte Träger für die Entfernung von Ni und Co. Auch LISTER[99] drückt so die Verunreinigungen von Zn-, Cd- und Bi-Salzen mit Eisen auf unter 2 p.p.m. herunter.

GAPON[54, 55] kombiniert die Reinigung mit Al_2O_3, ZnO und ZnS für die Herstellung von Phosphoren, GURVICH[59] verwendet dazu noch Dimethylglyoxim.

FISCHER[33] konnte Yttrium auf einen Gehalt von weniger als $2 \cdot 10^{-3}\%$ seltene Erden bringen.

11. *Verwendung von Komplexlösungen*

Häufig eignen sich Lösungen von Komplexen gut für Trennungen. Die Komplexbildung verhindert sicherlich oft die hydrolytische Fällung eines Teiles oder aller

Kationen, sodass diese dementsprechend gereiht adsorbiert werden. Welchem Fixierungs-Mechanismus, die als Komplexe gebundenen Metalle gehorchen, bleibt meist unklar.

SCHWAB[155] stellt fest, dass die Reihung der Kationen durch Überführung in Ammine oder Tartratkomplexe vollkommen geändert wird. Halbmetalle wie As und Sb, welche auf die Al_2O_3-Säule als Chloride aufgebracht, sofort vollkommen hydrolysiert werden, können überhaupt nur in komplexer Form chromatographiert werden, vor allem als Tartrate. ERÄMETSÄ[21, 22] verwendet ebenfalls Tartrate und auch Citrate zur Komplexbildung der seltenen Erden an Al_2O_3. PINTEROVIC[140] trennt als Tartrate die Ionen von As, Sb, Bi und Sn, ebenso KARSCHULIN[75].

KORENMAN[83] beschreibt einen Mikronachweis von Cadmium mit Hilfe der Cyankomplexlösung an SiO_2.

TIKHOMIROFF[192] trennt Tantal von Niob von der Al_2O_3-Säule durch Elution mit Ammonoxalat. SENYAVIN[171] verwendet die Elution mit verschiedenen Komplexbildern zur Feststellung der Komplexstabilität, ähnliche Vergleiche stellt GURVICH[60] mit Dimethylglyoximkomplexen an.

LISTER[98] vergleicht die Reihung bei Anwendung der Komplexbildner NH_3 und Tartrat mit der in rein wässriger und Dioxan-hältiger Lösung, welche alle verschieden erscheinen und zieht Rückschlüsse auf den Dissoziationsgrad der Salze. JANKOW[74] verwendet verschiedene Komplexbildungen zur Unterstützung der Trennfähigkeit der Säule, so CNS^- und F^- für Eisen. OKAC[128, 129] arbeitet mit Lösungen komplexer Thiosalze und Cyanide auf imprägniertem Papier und verwendet die Ringchromatographie für diese Trennungen.

12. *Radiochemische Methoden*

Die Verwendung von Radioindikatoren auf der Al_2O_3-Säule hat LINDNER[92] mit aktivem Blei, Wismut, Barium und Radium eröffnet. Seine weiteren Arbeiten betreffen vor allem die Trennung der seltenen Erden[93–97], doch reichen seine Ergebnisse nicht an die mit Ionenaustauscherharzen erzielten heran. Die Adsorption von Cu^{2+} und Zn^{2+} an $CaCO_3$ hat CANALS[11] chromatographisch nach der tracer-Methode untersucht.

Von Arbeiten in dieser Richtung seien noch Versuche von GAPON[51] mit $^{32}PO_4^{3-}$ an Al_2O_3, von OLSHANOVA[135] über Adsorption und Desorption von Kationen an Al_2O_3 und von KOPYLOVA[82] über die Trennungsmöglichkeiten bei der Fällungschromatographie hervorgehoben.

13. *Spezielle analytische Methoden*

Die Eignung des Al_2O_3 für die Trennung ähnlicher Ionen wurde mehrfach untersucht. So hat SCHWAB[164] die Platinmetalle getrennt und auch VENTURELLO[203] hat dasselbe Problem mit Erfolg bearbeitet.

Die seltenen Erden haben ERÄMETSÄ[21, 22] und CROATTO[14, 15] an Al_2O_3 zu trennen

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versucht, erhielten aber keine guten Ergebnisse, wie oben erwähnt auch nicht Lindner[93-97], hingegen hatte Fischer[30a, 33] in Kombination anderer Verfahren damit gute Erfolge zu verzeichnen. Hansen[61, 62] trennt Zirkon von Hafnium auf SiO_2 wobei der Zusatz von Methanol offen lässt, ob es sich um Verteilungschromatographie oder molekulare Adsorption handelt.

Als Hilfsmittel für quantitative Bestimmungen wird die Al_2O_3-Säule von Dean[19] für die Entfernung von F^- und SO_4^{2-}, von Nydahl[122] für letzteres und HPO_4^{2-} verwendet. Vichutinskii[207] entfernt PO_4^{3-} bei der Analyse von Pflanzenasche mit der Al_2O_3-Säule.

Olshanova[130-134] gibt qualitative Analysengänge mit wesentlicher Verwendung einer Al_2O_3-Säule. Bishop[7] und Fillinger[28] geben Anregungen für die Einführung der anorganischen Chromatographie in den qualitativen Lehrgängen.

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PHÉNOMÈNES IONIQUES MODIFIANT LE PARTAGE DANS LA CHROMATOGRAPHIE SUR PAPIER

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Les différents facteurs impliqués dans la chromatographie sur papier ont été analysés par MARTIN[1] qui distingue le partage vrai, prédominant dans la plupart des cas, de facteurs secondaires tels que l'adsorption[2] ou l'échange d'ions dû aux carboxyles du papier[3] dont l'importance peut être exaltée dans certaines circonstances. Sans aborder ici les problèmes liés à l'adsorption, nous rapporterons quelques expériences montrant l'influence de l'état d'ionisation des substances chromatographiées sur l'allure du phénomène de partage.

MATÉRIEL ET TECHNIQUES

La chromatographie a été réalisée sur l'extrait acétique d'un broyat de muscles de scorpions (*Androctonus australis* L.)[4] suivi d'un dessalage à l'alcool et de concentration sous vide jusqu'à consistance sirupeuse.

Les composés guanidiques témoins: arginine, agmatine, arcaïne, méthylguanidine et l'acide γ-guanidobutyrique provenaient de Hoffmann-La Roche.

Les solutions d'indicateurs nucléaires renfermaient:

(1) ^{22}Na (A.S.: 1 mC/mmole) contenu soit dans la solution d'origine renfermant 0.039 μéquiv./μl de ^{22}NaCl et 0.039 μéquiv./μl de HCl, soit dans la même solution exactement neutralisée à pH = 7.0 et contenant 0.044 μéquiv/μl de ^{22}NaCl d'A.S.: 0.5 mC/mmole.

(2) ^{36}Cl (A.S.: 0.001 mC/mmole) utilisé soit sous la forme de la solution d'origine: H^{36}Cl (0.58 μéquiv./μl) soit sous celle d'une solution neutralisée par NaOH et contenant 0.36 μéquiv./μl de Na^{36}Cl.

Des volumes de 1 à 30 μl de ces solutions ont été utilisés pour la chromatographie sur papiers Whatman No. 1 ou 4 préalablement lavés ou non selon la technique de KAY, HARRIS ET ENTENMAN[5] par capillarité descendante dans une cuve de verre.

Les solvants utilisés ont été: *n*-butanol–acide acétique–eau (78:5:17 et 73:10:17) (BA 5 et BA 10); pyridine–alcool isoamylique–acide acétique–eau (80:40:10:40) (Pyridine); eau bidistillée dans un appareil entièrement en verre Pyrex.

La révélation des chromatogrammes a été réalisée: (a) soit avec des indicateurs de pH: vert de bromocrésol (VB) (0.04% dans l'éthanol à 95°), hélianthine (0.02% dans l'éthanol à 60°), rouge de phénol (0.02% dans l'éthanol à 95°), (b) soit avec des réactifs spécifiques: de SAKAGUCHI[6] pour les dérivés guanidiques monosubstitués, $AgNO_3$ 0.1 *N* pour Cl^-, acétate d'uranyle et de zinc selon FEIGL[7] pour Na^+ et forma-

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tion de phosphomolybdate réduit pour PO_4^{3-} selon Pollard et McOmie[8], (c) soit par autoradiographie des chromatogrammes avec des films X-ray no screen Kodak.

RÉSULTATS

Le chromatogramme de l'extrait naturel de muscle de scorpions, riche en sels, réalisé en butanol–acétique et révélé au réactif de Sakaguchi montre (Fig. 1), à côté de spots ovalaires dont les R_F sont très voisins de ceux des composés de référence, des taches en forme de V traduisant une perturbation du processus de chromatographie.

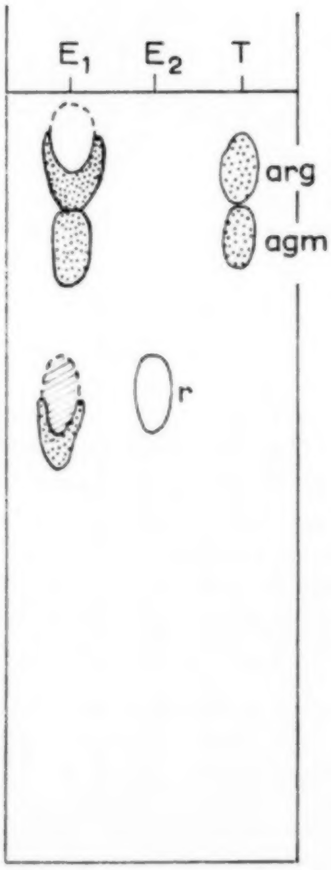

Fig. 1. Schéma d'un chromatogramme en BA10 d'un extrait naturel de post-abdomen de scorpions (voir texte). E_1: révélation vert de bromocrésol (les zones hachurées sont bleues) et au réactif de Sakaguchi (zones pointillées roses). E_2: révélation au rouge de crésol (r = rouge). T: témoins, arg = arginine, agm = agmatine.

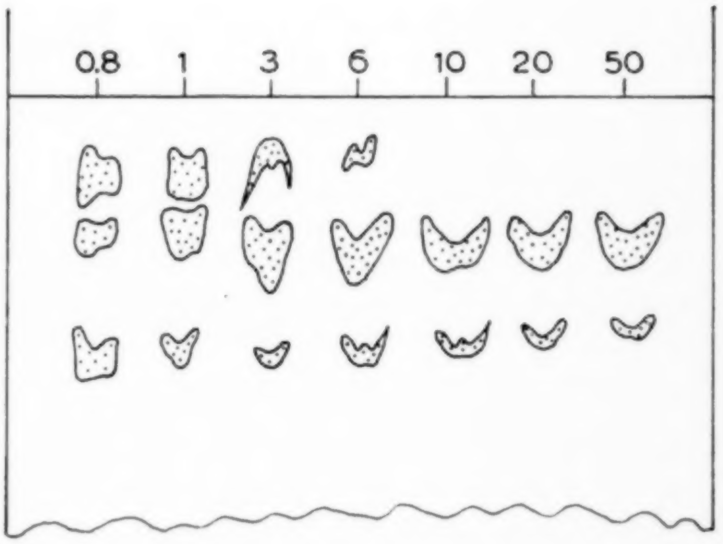

Fig. 2. Schéma d'un chromatogramme en BA10 de 5 μg d'agmatine supplémentés avec des quantités croissantes de NaCl (0.8, 1, 3, 6, 10, 20 et 50 μéquiv.). Papier Whatman 4 lavé, temps de migration: 20 h. Révélation: réactif de Sakaguchi.

La plus caractéristique est une tache en V (Sakaguchi +, $R_F = 0.20$) ne correspondant à aucun des dérivés guanidiques connus. L'électrophorèse sur papier (tampon véronal de pH = 8.6) ou la chromatographie en Pyridine ne distinguent, cependant, en rien ce produit de l'agmatine témoin. Inversement, de l'agmatine additionnée à l'extrait se comporte comme la tache inconnue. Les révélations aux indicateurs de pH montrent la présence d'un spot basique épousant exactement à sa partie inférieure la forme du spot d'agmatine et, réciproquement, l'addition de soude à une solution d'agmatine donne les mêmes images en butanol–acétique. Les sels neutres (chlorures et sulfates alcalins) produisent eux aussi des troubles évidents dans la migration des

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guanidiques. Ainsi la chromatographie d'agmatine en présence de quantités croissantes de NaCl révèle la présence de 2 et 3 spots d'agmatine (Fig. 2). Les indicateurs de pH montrent qu'il y a apparition de plages acides, alcalines ou neutres tout au long du chromatogramme. Les Figs. 3, 4 et 5 montrent des chromatographies de sels neutres et basiques mettant en évidence ces discontinuités de pH en cours de migration. La Fig. 6 montre qu'un sel organique (chlorhydrate de méthylguanidine) est capable de donner une décomposition encore plus nette.

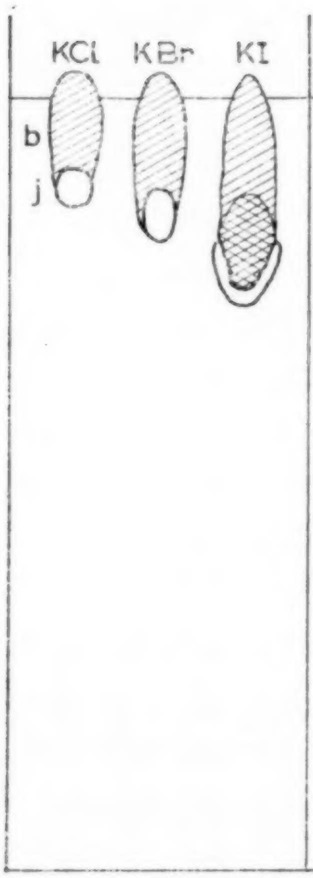

Fig. 3.

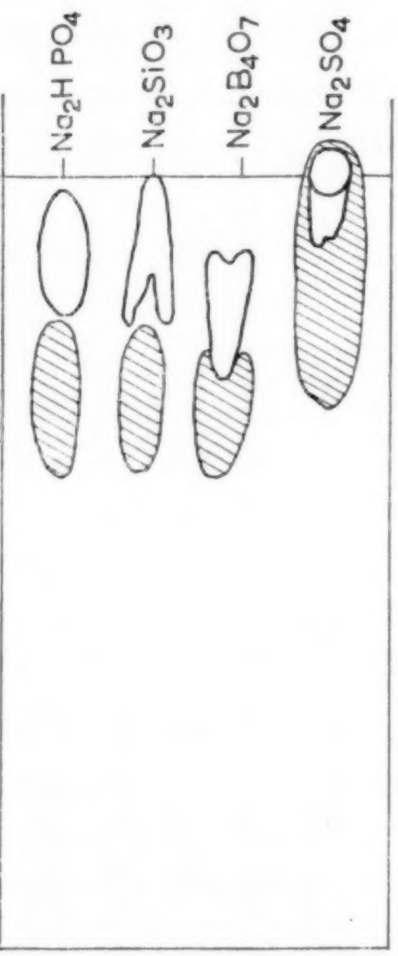

Fig. 4.

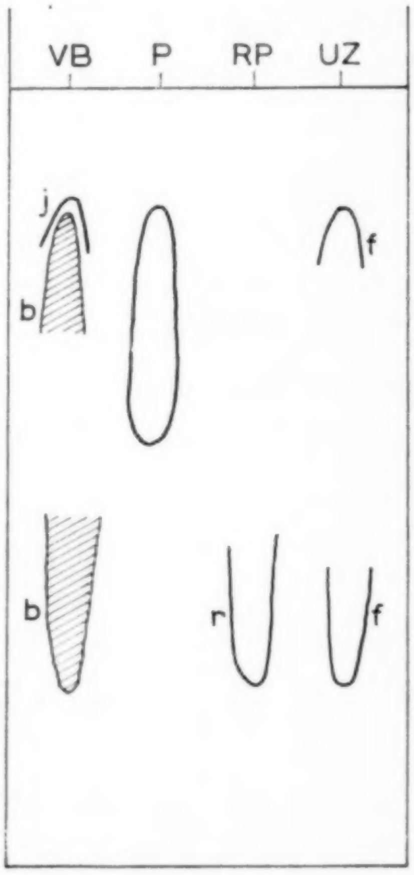

Fig. 5.

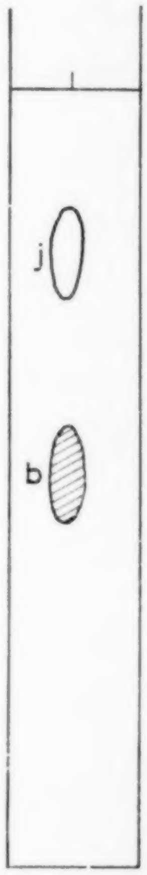

Fig. 6.

Fig. 3. Schéma d'un chromatogramme en BA10 de KCl, KBr et KI (10 μl d'une solution saturée). Papier Whatman 1, temps de migration: 14 h. Révélation au vert de bromocrésol. Les zones hachurées sont bleues (b), les incolores jaunes (j).

Fig. 4. Même expérience avec Na_2HPO_4, Na_2SiO_3, $Na_2B_4O_7$ et Na_2SO_4 (10 μl d'une solution saturée).

Fig. 5. Schéma d'un chromatogramme en BA10 de Na_2HPO_4 (1 μéquiv.). Révélations au vert de bromocrésol (VB), à l'acide molybdique (P), au rouge de phénol (RP) et à l'acétate d'uranyle et de zinc (UZ). j = jaune; b = bleu; r = rouge; f = fluorescence jaune-verte en lumière U.V.

Fig. 6. Schéma d'un chromatogramme en BA10 de chlorhydrate de méthylguanidine (5 μg). Mêmes conditions expérimentales que pour la Fig. 3. La tache b est bleue au VB et rose au réactif de SAKAGUCHI (acétate de méthylguanidine); la tache j, jaune au VB (HCl).

Le désir de réaliser des perturbations importantes en faisant migrer des composés ionisés sur une grande longueur de papier nous a incité à utiliser la migration dans l'eau; on peut ainsi obtenir une séparation rapide et correcte de quelques dérivés guanidiques (Fig. 7). De même, on observe dans l'eau une décomposition spectaculaire de sels minéraux, source de perturbations pour la caractérisation de composés orga-

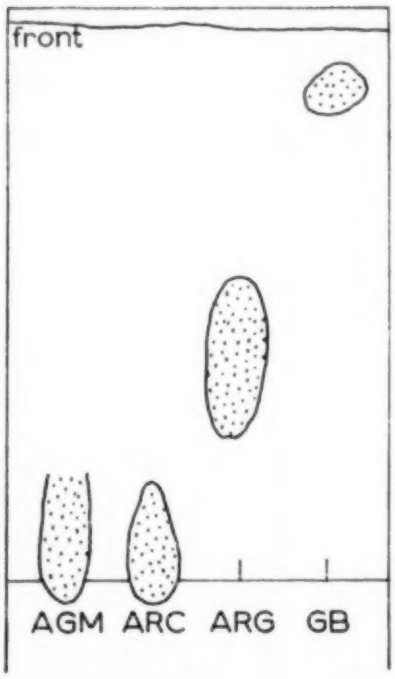

Fig. 7. Schéma d'un chromatogramme (ascendant) dans l'eau bidistillée d'agmatine (agm), arcaïne (arc), arginine (arg) et acide guanidobutyrique (GB), 10 μg. Papier Whatman 20 non lavé. Révélation au réactif de SAKAGUCHI.

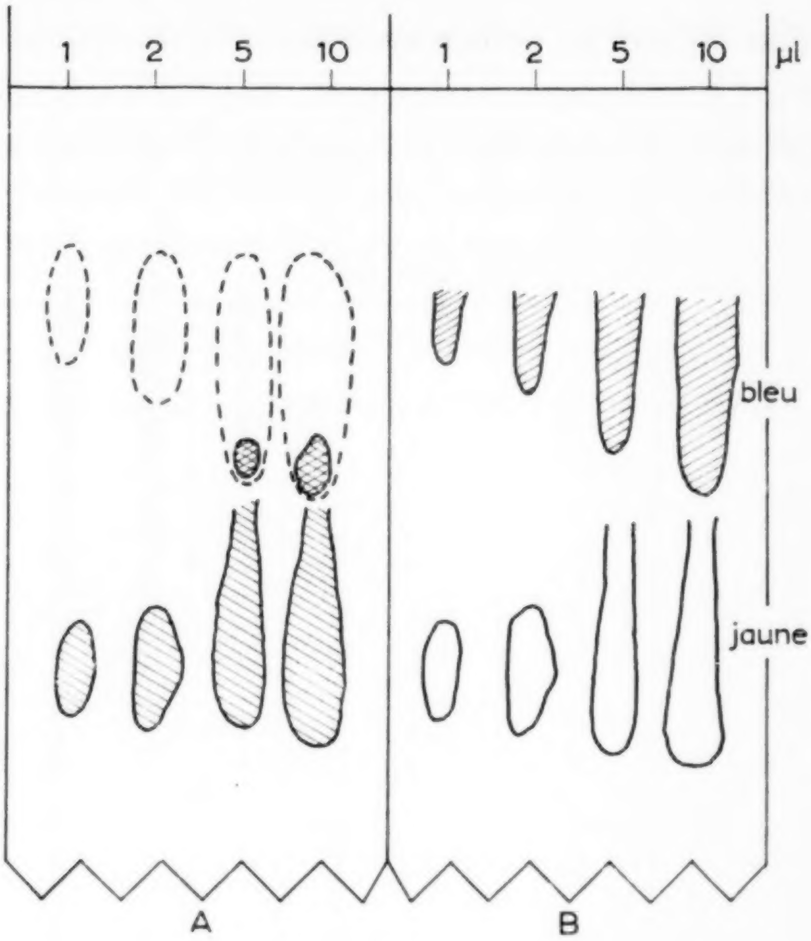

Fig. 8. Schéma d'un chromatogramme (descendant) en BA5 d'une solution de ^{22}NaCl neutre (0.044 μéquiv/μl). Papier Whatman 4 lavé. Migration: 4 jours. A: révélation au nitrate d'argent 0.1 *N*. Les pointillés correspondent aux zones impressionnées en autoradiographie. B: révélation au vert de bromocrésol. Les chiffres indiquent le nombre de microlitres déposés.

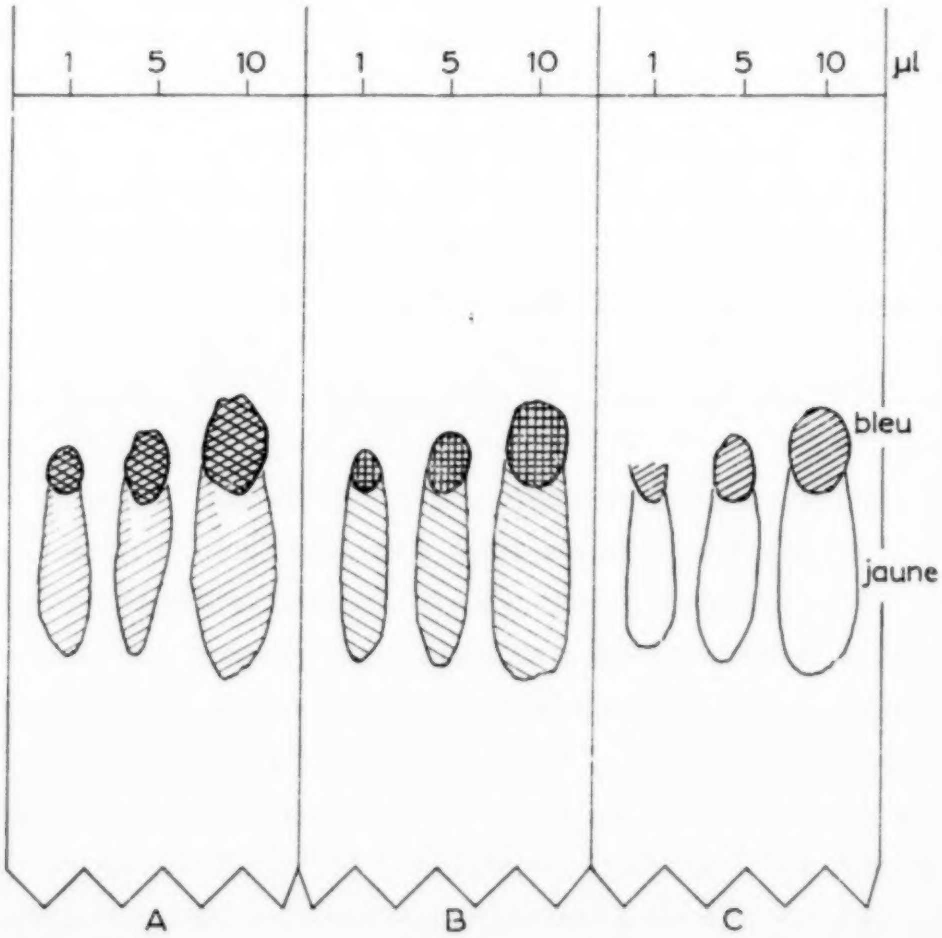

Fig. 9. Même expérience que pour Fig. 8 mais avec Na^{36}Cl (solution neutre à 0.36 μéquiv./μl). A: autoradiogramme. B: révélation au nitrate d'argent. C: révélation au vert de bromocrésol.

niques dans un milieu complexe. Cette décomposition a été objectivée en utilisant NaCl doublement marqué par ^{22}Na et ^{36}Cl, le recours à l'emploi de traceurs ayant été initialement pratiqué par LEDERER[9] et STRAIN ET MURPHY[10]. Les Figs. 8, 9, et 10

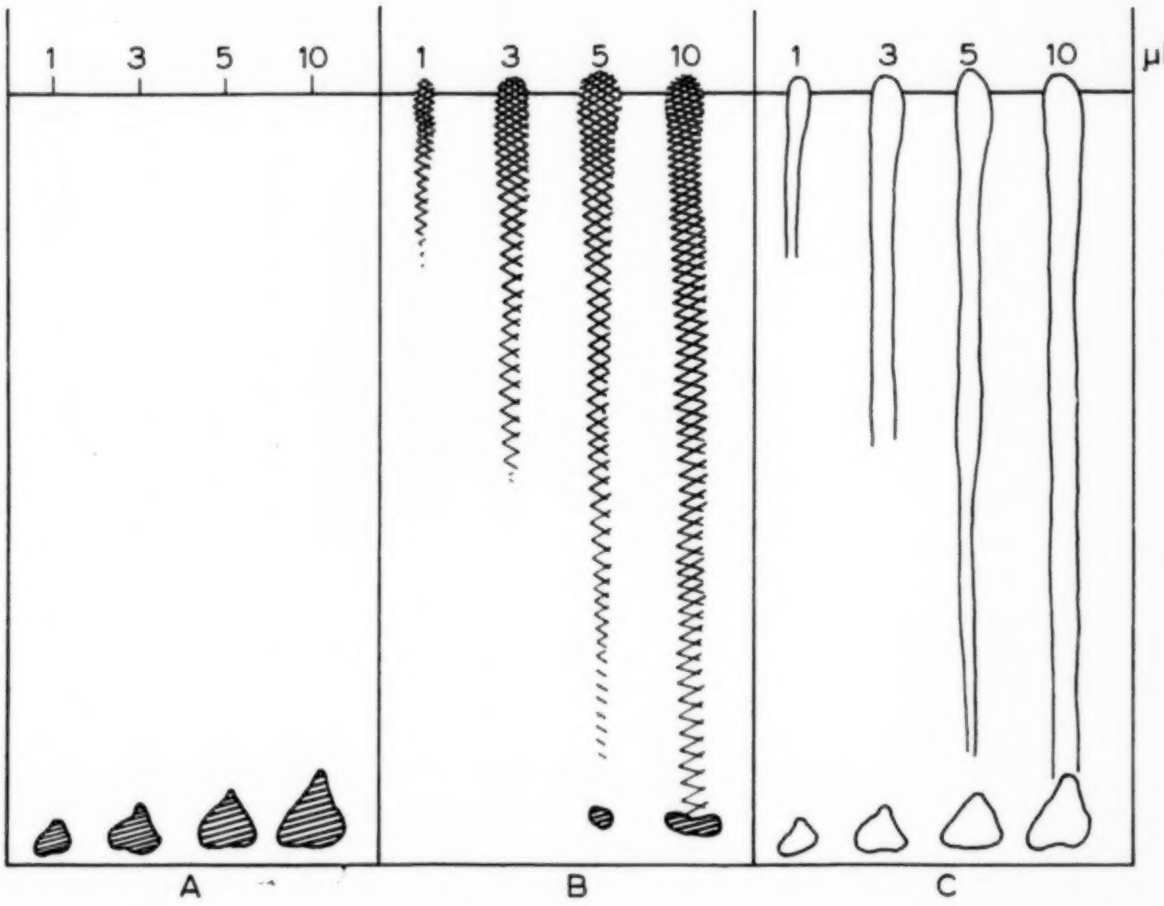

Fig. 10. Schéma d'un chromatogramme (descendant) en eau bidistillée; papier Whatman 4 lavé; migration: 2 h. A: d'une solution Na^{36}Cl (0.36 µéquiv./µl). Révélation par autoradiographie. B: d'une solution ^{22}NaCl (0.044 µéquiv./µl). Révélation par autoradiographie. Traînée correspondant au Na^{+} échangé par le papier. Les taches en front pour les essais 5 et 10 µl correspondent au NaCl non dissocié. C: d'une solution de Na^{36}Cl: révélation au vert de bromocrésol, AgNO$_3$ et hélianthine, la tache en front étant respectivement jaune, gris-noire et rose avec ces réactifs.

montrent l'importance de la dissociation obtenue pour cet électrolyte, ce qui permet d'expliquer que la séparation dans l'eau des dérivés guanidiques soit complètement bouleversée par la présence de sels neutres comme NaCl ou Na_2SO_4 (Fig. 11).

DISCUSSION

Les anomalies observées dans ces expériences peuvent toutes s'expliquer par des phénomènes ioniques modifiant le partage, l'intérêt des composés guanidiques étant de constituer de véritables indicateurs de chromatographie parce que très sensibles à ces perturbations. L'agmatine en particulier (base en C_5 non amphotère) passera facilement de l'état ionisé à celui de base non dissociée pour une faible augmentation de la concentration en ions OH^-, ce changement d'état impliquant des différences considérables du coefficient de partage; la forme non ionisée étant beaucoup plus organophile. Ceci est d'autant plus important sur le plan pratique que la purification sur résines échangeuses d'ions d'extraits naturels contenant des bases organiques relativement fortes du type de l'agmatine est à peu près impossible à obtenir à cause

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du pK élevé de ces bases en comparaison de celui de bases volatiles comme NH_4OH servant d'éluant.

Les perturbations ioniques en chromatographie peuvent avoir comme origine: le papier, le solvant ou les électrolytes présents dans la solution soumise à la chromatographie. L'interprétation de nos résultats peut être réalisée en fonction de ces trois facteurs.

En tant qu'échangeur d'ions[3, 11, 12], le papier peut jouer un rôle direct en participant à la séparation des composés, ce qui paraît permettre d'interpréter au moins partiellement les séparations obtenues dans l'eau pure (Fig. 7) bien que certaines observations[13, 14] ne puissent l'être de cette manière.

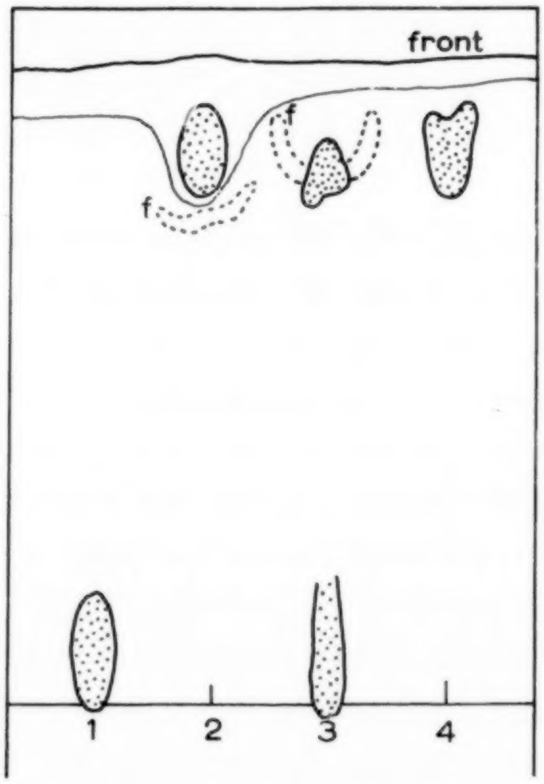

Fig. 11. Schéma d'un chromatogramme dans l'eau bidistillée (temps de migration: 2 h, ascendant) de 5 μg d'agmatine (1), supplémentés de 10 μéquiv. de Na_2SO_4 (2), 10 μéquiv. de NaCl (3) et 20 μéquiv. de NaCl (4). Révélation: réactif de SAKAGUCHI. f = zone fluorescente blanc-bleuâtre en lumière U.V. due aux sels.

Le solvant organique constituant la phase mobile est souvent chargé de composés ionisables (acides ou basiques) et leur présence, en excès par rapport aux substances à chromatographier, conditionne la séparation de ces dernières sous forme de sel de l'acide ou de la base contenu dans le solvant. Dans cet ordre d'idées, WESTALL[15] et CONSDEN ET GORDON[16] appliquant la loi d'action des masses au cas du phénol saturé d'eau et du butanol acétique, expliquent les décompositions salines survenues dans ces solvants.

En ce qui concerne les électrolytes, on a envisagé des interactions de type ionique ou autre entre les substances soumises à la chromatographie, par exemple entre acides aminés[17] (voire entre sucres[18]), mais ces phénomènes restent mal expliqués. Par contre, un type d'interactions facile à concevoir est celui de la chromatographie d'extraits naturels où l'on est amené à utiliser des solutions riches en sels. S'ils ont une dissociation basique, cas de notre extrait, leur présence explique la forme en V du spot d'agmatine (Fig. 1), base non amphotère passant en milieu alcalin sous forme non ionisée dont la solubilité pour le butanol est beaucoup plus grande: de ce fait, l'agmatine ne pourra se déposer qu'à la limite du spot alcalin dû aux sels, là où elle pourra reprendre sa forme d'ion agmatinium beaucoup plus soluble dans l'eau*. Les sels neutres peuvent modifier la chromatographie en rendant le produit analysé moins soluble dans la phase aqueuse (relargage) ou par démixion du solvant à leur niveau[20]. A côté de ces effets, l'influence des carboxyles du papier paraît devoir jouer un rôle non négligeable par fixation du cation du sel (Figs. 8 à 10). Les conséquences de ces dissociations sont illustrées par les Figs. 2 et 11. Sur cette dernière, on voit le déplacement spectaculaire dans l'eau de l'agmatine, qui, normalement collée au départ, migre pratiquement en front en présence de NaCl ou Na_2SO_4. Ce déplacement peut être expliqué par son décrochage du papier par le sodium qui s'échangerait préférentiellement et aussi parce qu'elle serait solubilisée dans l'eau par HCl provenant de la dissociation du sel.

La Fig. 2 montre des images plus complexes données par la chromatographie de l'agmatine en butanol–acide acétique en présence de quantités croissantes de NaCl. En partant du front, on trouve un spot en forme de V, de surface à peu près constante, correspondant au déplacement de l'agmatine par l'action alcalinisante de l'acétate de sodium formé par hydrolyse de NaCl et dont la quantité est sensiblement proportionnelle à la surface de papier parcourue; un autre spot en V, mais plus important, dont la superficie croît avec la concentration en NaCl, sa forme pouvant s'expliquer par un effet de relargage du sel vis-à-vis de l'agmatine; enfin, un spot en V renversé qui va s'amenuisant pour disparaître à partir d'une certaine concentration en NaCl et dont l'existence s'explique facilement si l'on admet que, lors du dépôt, on a réalisé une chromatographie circulaire ayant eu pour effet de déposer l'agmatine à la périphérie de l'élément perturbateur (NaCl). L'importance des perturbations est conditionnée par la nature des sels et leur concentration. Cette dernière constitue un facteur prépondérant puisque la dimension d'un spot dépend de la quantité de produit présente au départ: en conséquence, le R_F d'une substance venant se déposer à la limite du spot des sels dépend directement de la concentration de ceux-ci. Si, par exemple, dans l'extrait naturel que nous avons utilisé, l'agmatine a un R_F de 0.20, elle aurait un R_F différent dans un autre extrait où la concentration en sels n'aurait pas été la même. Nous avons pu ainsi faire varier le R_F de l'agmatine de 0 à 0.50 dans des expériences réalisées avec différents sels à différentes concentrations.

CONCLUSION

Le caractère de base non amphotère de l'agmatine, composé réagissant d'une manière sensible aux variations de pH (alors que les amphotères peuvent s'adapter assez facilement à ces perturbations) a permis de mettre l'accent sur l'allure hétérogène de la chromatographie d'un extrait naturel riche en sels, en utilisant ce composé comme un véritable indicateur. Travaillant dans une direction opposée par rapport à des auteurs comme MUNIER[20] et MACFARREN[21] qui ont cherché à éviter les phénomènes de déséquilibre pour améliorer la séparation de composés organiques, nous avons voulu mettre à profit des conditions défavorables imposées par l'expérience pour essayer de préciser certains mécanismes impliqués dans la chromatographie sur papier.

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* La formation en milieu alcalin, d'agmatine base non dissociée fournit l'explication théorique de l'utilisation des papiers imprégnés de NaOH 0.2 N employés pour la séparation des bases guanidiques[19]. Cette méthode permet de séparer les bases non amphotères (agmatine, arcaïne) transformées en composés non dissociés, de R_F quintuplé, des dérivés amphotères dont le R_F est beaucoup moins modifié: doublé pour l'arginine et même diminué pour la glycocyamine dont le pK_1 est le plus bas.

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RÉSUMÉ

Voulant réaliser la séparation de dérivés guanidiques mono-substitués présents dans des extraits naturels et utilisant pour la révélation: réactifs spécifiques, réactifs de pH et autoradiographie, nous avons étudié, à l'occasion des perturbations observées, le rôle respectif des différents facteurs susceptibles d'apporter des modifications ioniques au milieu: papier, solvant et électrolytes présents dans la solution soumise à la chromatographie.

SUMMARY

In order to separate the mono-substituted guanidino-derivatives present in natural extracts, an investigation was made of the behaviour of these substances in paper chromatography. Specific reagents, pH indicators and autoradiography were used to identify the spots. The disturbances observed led the authors to study the role of various factors that are likely to produce ionic changes in the medium, namely the paper, the solvent and the electrolytes present in the solution subjected to chromatography.

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METALLIC NITRATES IN PAPER CHROMATOGRAPHY

V. SYSTEMS CONTAINING AMMONIUM THIOCYANATE

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In previous papers the chromatographic behaviour of a large number of metallic nitrates was reported: in mixtures of nitric acid and methyl isobutyl ketone[1]; in these same mixtures with the addition of lithium nitrate as a salting-out agent[2]; in the system di-isopropyl ether–nitric acid–water[3]; and finally in the system isoamyl alcohol–nitric acid–water[4].

In all the above reports the extent to which nitric acid (and lithium nitrate) are distributed between the aqueous and organic phases was determined. The effects were considered which this distribution had on the R_F values of metallic nitrates in each of these systems.

We believe that, up to the present, no attention has been paid to the exact composition of the eluants used in paper chromatography. A study of the distribution of the acid and the salting-out agent between the initial aqueous solution and the organic layers, and the corresponding R_F values, permitted a more fundamental interpretation of the R_F values obtained and served as a basis for some theoretical considerations.

Apart from being employed for numerous useful separations, partition chromatography on paper also yields results which may to a certain extent be related to the distribution coefficients of the same cations, in the same solvent system, obtainable by the extraction technique. Thus, a chromatographic study may serve as a preliminary to an investigation of the possibilities of separating larger quantities by extraction. It is important for this purpose to know the R_F values of as many ions as possible in the given solvent system.

With this aim in mind we undertook a comprehensive study of a large number of metallic nitrates in systems containing an inorganic complexing agent: ammonium thiocyanate. Chromatograms were run in: (1) methyl isobutyl ketone (MIBK), (2) di-isopropyl ether (DIPE), and (3) isoamyl alcohol (IAA), equilibrated with aqueous solutions containing various concentrations of the complexing agent both with and without nitric acid (approximately 0.1 *M*).

Useful R_F values were only obtained with the three following systems:

1. MIBK–HNO_3–NH_4CNS–H_2O
2. IAA–HNO_3–NH_4CNS–H_2O
3. IAA–NH_4CNS–H_2O

For the other three systems:

1. MIBK–NH_4CNS–H_2O
2. DIPE–HNO_3–NH_4CNS–H_2O
3. DIPE–NH_4CNS–H_2O

the R_F values of nearly all the cations tested were equal, or approximately equal, to zero.

The first three valuable solvent systems were then systematically studied by determining the distribution of the thiocyanate and nitric acid between the aqueous and organic layers. The distribution coefficients were then used to interpret the results obtained in the paper-chromatographic experiments.

Ammonium thiocyanate, owing to its great tendency to form complexes, has been widely used for the separation of cations by the extraction technique[5]. In paper-chromatographic separations advantage has also been taken of this tendency, and in recent years numerous procedures have been suggested[6]. LEDERER[7] studied extensively the possibilities of separating the rare earths by paper chromatography, using butanol saturated with thiocyanate as the eluant.

This distribution study describes the chromatographic behaviour of various elements under different experimental conditions. Although the results are not presented as established analytical procedures, they should be of considerable help in further development of analytical separations.

EXPERIMENTAL

The organic solvents, methyl isobutyl ketone, di-isopropyl ether and isoamyl alcohol were purified as previously described[2, 3, 4]. The initial aqueous solutions of ammonium thiocyanate were prepared by diluting a stock solution of Baker's Analyzed C.P. grade thiocyanate; for the solutions containing nitric acid non-fuming nitric acid was used. Solutions of the radioisotopes ^{144}Ce, ^{154}Eu, ^{153}Gd, ^{170}Tm and ^{106}Ru in dilute nitric acid, and ^{99}Tc were obtained from Harwell.

The distribution of the solutes in both aqueous and organic layers was followed potentiometrically for nitric acid as described in previous papers[1–4] and colorimetrically for thiocyanate using the ferric iron reaction.* The sum of the masses in both layers showed a maximum variation of ± 1% for nitric acid and ± 3% for thiocyanate. Good reproducibilities were obtained in the determination of the volume changes of the aqueous and organic phases after equilibration by the method previously given[3, 4]. The distribution of the solutes is presented as their normalities in both layers, as their concentration distribution coefficient α and as their mass distribution coefficient μ. The relation between these two coefficients is given by $\mu = (V_o/V_w)\cdot\alpha$, where V_o and V_w represent the volumes of the organic and aqueous layers after equilibration.

The technique and procedure for the chromatographic experiments, as well as the reagents and methods for detection of the spots have been described in previous papers[4, 8]. Radioisotopes were detected using either a Victoreen 1B85 thyrode counter tube with thin metal wall or a Nuclear Instrument, model D 34 mica end-window tube, employing the technique previously used[9].

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* The determinations were made in fresh solutions in order to eliminate the errors caused by the reaction between the nitric acid and the thiocyanate. However, no marked influence of this reaction upon the R_F values was observed.

The chromatographic experiments were carried out with the following solutions spotted on filter paper:

KNO_3
$RbNO_3$
$CsNO_3$
$Be(NO_3)_2$
$Mg(NO_3)_2$
$Ca(NO_3)_2$
$Sr(NO_3)_2$
$Ba(NO_3)_2$
$Cu(NO_3)_2$
$AgNO_3$
$Zn(NO_3)_2$
$Cd(NO_3)_2$
$Hg(NO_3)_2$
$Y(NO_3)_3$
$La(NO_3)_3$
$Ce(NO_3)_3$
$Pr(NO_3)_3$
$Nd(NO_3)_3$
$Sm(NO_3)_3$
$Eu(NO_3)_3$
$Gd(NO_3)_3$
$Er(NO_3)_3$
$Tm(NO_3)_3$
$Th(NO_3)_4$
$UO_2(NO_3)_2$
$Al(NO_3)_3$
$Ga(NO_3)_3$
$In(NO_3)_3$
$TlNO_3$
$Tl(NO_3)_3$
$ZrO(NO_3)_2$
$Pb(NO_3)_2$
$Bi(NO_3)_3$
$Cr(NO_3)_3$
$Mn(NO_3)_2$
$Fe(NO_3)_3$
$Co(NO_3)_2$
$Ni(NO_3)_2$
$Ru(NO_3)_3$
$AuCl_3$
$TiCl_3$
GeO_2
$SnCl_2$
VCl_3
NbF_5
TaF_5
$AsCl_3$
$SbCl_3$
NH_4MoO_4
Na_2WO_4
Na_2SeO_3
Na_2TeO_3
NH_4TcO_4
$KReO_4$
NaI
OsO_4
$RhCl_3$
$PdCl_2$
H_2PtCl_4

RESULTS AND DISCUSSION

Systems with isoamyl alcohol

Table I shows the normality of ammonium thiocyanate both in the initial aqueous solution, and in the aqueous and organic phases after equilibration, as well as the calculated distribution coefficients. Table II shows parallel results for the thiocyanate partition and the distribution of acid for the system containing 0.1 *M* nitric acid and various amounts of ammonium thiocyanate. As can be seen from Table I, the distribution coefficient α remains practically unchanged and is independent of the initial concentration of the thiocyanate for ammonium thiocyanate concentrations up to approximately 2 *M*. For higher initial concentrations a slight increase in α is observed. Table II shows results that are very similar. The distribution coefficient of

TABLE I

THE DISTRIBUTION OF AMMONIUM THIOCYANATE BETWEEN WATER AND ISOAMYL ALCOHOL

No. of soln.	*Normality of NH_4CNS*			V_o/V_w	α	μ
	initial soln.	*aqueous phase*	*organic phase*			
1	*2*	*3*	*4*	*5*	*6*	*7*
1	0.045	0.045	0.0031	1.12	0.069	0.077
2	0.090	0.091	0.0056	1.12	0.062	0.069
3	0.180	0.181	0.012	1.12	0.066	0.074
4	0.27	0.27	0.02	1.10	0.074	0.081
5	0.45	0.43	0.03	1.11	0.070	0.077
6	0.90	0.86	0.06	1.11	0.070	0.077
7	1.90	1.80	0.21	1.14	0.117	0.133
8	2.85	2.75	0.38	1.20	0.138	0.166
9	3.80	3.60	0.58	1.26	0.161	0.203
10	4.75	4.55	0.83	1.36	0.183	0.249

TABLE II

THE DISTRIBUTION OF AMMONIUM THIOCYANATE AND NITRIC ACID BETWEEN WATER AND ISOAMYL ALCOHOL

No. of soln.	*Normality in the initial solution*		*Normality in the aqueous phase*		*Normality in the organic phase*		V_o/V_w	*Distribution coefficients of*			
								HNO_3		NH_4CNS	
	HNO_3	NH_4CNS	HNO_3	NH_4CNS	HNO_3	NH_4CNS		α	μ	α	μ
1	2	3	4	5	6	7	8	9	10	11	12
1	0.098	0.00	0.096	0.00	0.005	0.00	1.08	0.06	0.065	—	—
2	0.097	0.45	0.038	0.39	0.059	0.072	1.09	1.55	1.70	0.187	0.204
3	0.097	0.90	0.024	0.82	0.072	0.12	1.14	3.00	3.42	0.145	0.165
4	0.097	1.90	0.011	1.70	0.081	0.26	1.17	7.36	8.64	0.153	0.179
5	0.097	2.85	0.009	2.61	0.081	0.44	1.21	9.00	10.90	0.166	0.202
6	0.097	3.80	0.006	3.59	0.081	0.63	1.29	13.50	17.50	0.175	0.228
7	0.097	4.75	0.005	4.35	0.082	0.79	1.31	16.40	21.50	0.181	0.237

the thiocyanate is only slightly influenced by a tenfold increase of the thiocyanate concentration in the initial aqueous solution. Furthermore, the presence of 0.1 *M* nitric acid has practically no influence on the passage of the thiocyanate from the aqueous solution into the alcohol.

As regards the distribution coefficient of the hydrogen ion, the opposite behaviour is observed. When no thiocyanate is present in the solution, only traces of the acid pass into the alcoholic phase, but a very small amount of thiocyanate increases the acid distribution 25-fold. To obtain further information regarding the acidic species extracted by the alcohol, we plotted the logarithm of $\alpha_{[H^+]}$ against the logarithm of

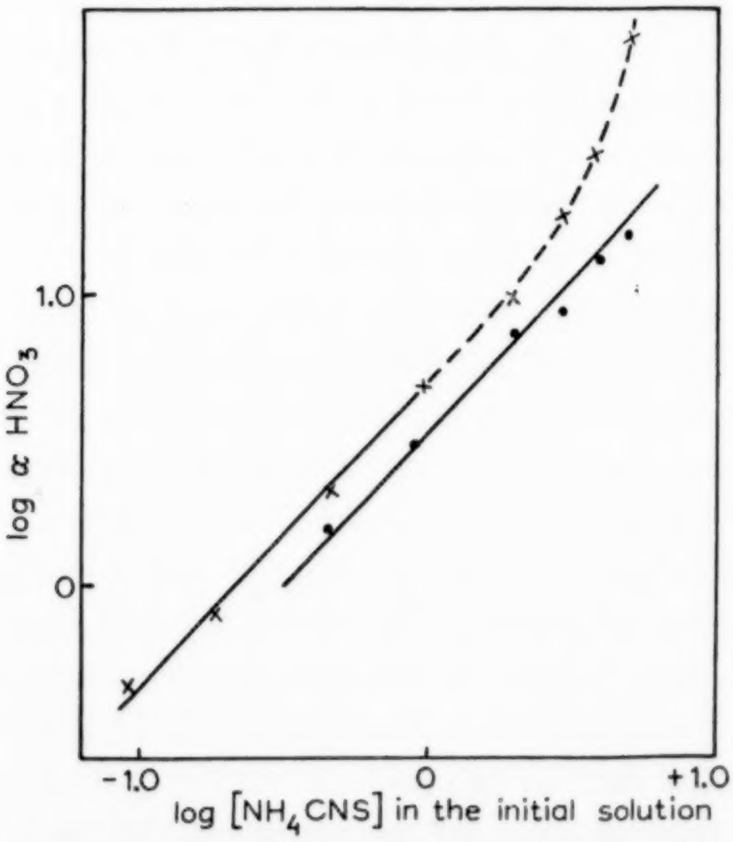

Fig. 1. The distribution coefficient of nitric acid (α_{HNO_3}) as a function of ammonium thiocyanate concentration in the initial aqueous solution. ● in the system IAA–NH_4CNS–HNO_3–H_2O. × in the system MIBK–NH_4CNS–HNO_3–H_2O.

the thiocyanate concentration in the initial aqueous solution (Fig. 1). A straight line was obtained with a slope = 1, indicating that each hydrogen ion of the nitric acid is attached to one thiocyanate. The extracted species, therefore, probably consist of free thiocyanic acid in addition to ammonium thiocyanate molecules.

The results obtained in paper-chromatographic experiments are presented in Table III. The ions not mentioned in the table gave R_F values equal to, or approximately equal to, zero in all the systems tested, except in the cases of molybdenum, vanadium and titanium. These cations show "multi-spot" phenomena, so that exact R_F values could not be given. The behaviour of these cations is complicated by the fact that they co-exist in two or more chemical forms, with widely different chromatographic behaviour, in a given solvent system. In addition to the "multi-spots" shown by these cations, a tailing was observed, due probably to a relatively slow rate of transition from one chemical form to another. Such phenomena are quite commonly observed in paper chromatography of these cations. The cations with R_F values of zero form either thiocyanates that are insoluble in water and alcohol, *e.g.* silver, thallium and lead, or thiocyanates or nitrates that are inextractable by alcohol, *e.g.* alkali and alkaline earth metals.

As regards the cations listed in Table III, the effect of increasing the thiocyanate concentration in the initial aqueous solution has first to be considered. In the systems without nitric acid all the cations, with the exception of arsenic, antimony, gold and palladium, show zero or nearly zero R_F values for concentrations of thiocyanate in the initial solution up to approximately 2 *M*. Higher R_F values in more concentrated solutions are connected with the appearance of the "water-front" on the chromatograms, the cations moving with the water-front. The appearance of the water-front must be due to a higher solubility of water in the alcohol, as can be seen from the volume ratios of the two separated phases (Table I, column 5). For concentrations up to 1.9 *M* thiocyanate in the initial aqueous solution, the ratio V_o/V_w is practically constant (± 1%); for higher initial concentrations, this ratio increases due to the swelling of the organic phase by its greater water content. The increase in the α_{NH_4CNS} coincides with the increase in the ratio V_o/V_w, as pointed out above.

Most of the cations listed in Table III readily form complex compounds with the thiocyanate, but it seems that they are only sparingly soluble in the alcohol. For a higher solubility (higher R_F values) a higher polarity of the solvent is needed. Greater polarity is achieved by increasing the water content of the solvent, *i.e.* by equilibrating it with a more concentrated aqueous solution of ammonium thiocyanate.

The above conclusions concerning the effect of initial thiocyanate concentration upon the R_F values obtained, seem to be completely valid for the parallel systems containing nitric acid. The "break" in the chromatographic behaviour of cations at the initial thiocyanate concentration of about 2 *M*, could be observed to a lesser degree, due to fewer experimental data being presented for lower thiocyanate concentrations.

The main aim of these experiments was to study the effect of the acid upon the R_F values in systems containing various concentrations of thiocyanate. Comparing the R_F values for the cations in the two sets of experiments, in the presence and

TABLE III

R_F VALUES OF IONS OBTAINED IN ISOAMYL ALCOHOL SATURATED WITH VARIOUS CONCENTRATIONS OF AQUEOUS AMMONIUM THIOCYANATE SOLUTION IN THE PRESENCE AND IN THE ABSENCE OF NITRIC ACID

M HNO_3	0									0.097						
M NH_4CNS	0.00	0.09	0.18	0.45	0.90	1.90	2.85	3.80	4.75	0.00	0.45	0.90	1.90	2.85	3.80	4.75
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
$Cu(NO_3)_2$	0.01	0.01	0.01	0.01	0.01	0.03	0.08 t	0.16	0.12	0.00	0.09	0.04	0.06	0.10	0.12	0.09
$AuCl_3$	0.49	0.12 t	0.12 t	0.13 t	0.11 t	0.22	0.26	0.29	0.32	0.23	0.64	0.66	0.73	0.77	0.82	0.84
$Be(NO_3)_2$	0.06	0.04	0.06	0.06	0.06	0.16	0.21	0.21	0.22	0.04 t	0.27 t	0.30 t	0.33 t	0.39 t	0.51	0.49
$Mg(NO_3)_2$	0.01	0.01	0.00	0.02	0.03	0.18	0.28	0.27	0.28	0.00	0.01	0.06	0.14	0.22	0.26	0.33
$Zn(NO_3)_2$	0.00	0.00	0.00	0.02	0.07	0.39	0.41	0.41	0.43	0.00	0.48 t	0.57 t	0.56 t	0.61 t	0.77	0.77
$Cd(NO_3)_2$	0.01	0.01	0.01	0.01	0.02	0.15	0.21	0.25	0.30	0.00	0.22	0.26	0.27	0.26	0.29	0.32
$Hg(NO_3)_2$	0.00	0.00	0.00	0.01	0.01	0.20	0.28	0.42	0.34	0.00	0.59	0.60	0.62	0.68	0.67	0.64
$Al(NO_3)_3$	0.00	0.00	0.00	0.00	0.01	0.09	0.13	0.10	0.05	0.03	0.08	0.10	0.21	0.27	0.38	0.41
$Ga(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.16				0.00	0.02	0.03	0.16	0.22	0.28	0.26
$In(NO_3)_3$	0.00	0.03	0.00	0.01	0.03	0.27	0.38	0.29	0.30	0.00	0.58 t	0.59 t	0.67 t	0.71 t	0.82 t	0.82 t
$Y(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.10	0.18	0.21	0.27	0.00	0.00	0.02	0.14	0.23	0.26	0.28
$La(NO_3)_3$		0.00	0.00	0.00	0.00					0.00	0.00	0.01	0.13	0.21	0.26	0.28
$Ce(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.10	0.17	0.27	0.21	0.00	0.00	0.06	0.17	0.26	0.28	0.28
$Pr(NO_3)_3$		0.00	0.00	0.00	0.00	0.14	0.21	0.26	0.30	0.00	0.00	0.03	0.15	0.23	0.27	0.27
$Nd(NO_3)_3$		0.00	0.00	0.00	0.00	0.07	0.16	0.19	0.24	0.00	0.01	0.04	0.15	0.23	0.27	0.28
$Sm(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.15	0.21	0.29	0.26	0.00	0.00	0.03	0.15	0.23	0.27	0.27
$Eu(NO_3)_3$		0.00	0.00	0.00	0.00	0.00	0.06	0.08	0.08	0.00	0.00	0.04	0.16	0.22	0.27	0.25
$Gd(NO_3)_3$		0.00	0.00	0.00	0.00	0.15	0.22	0.27	0.26	0.00	0.00	0.03	0.13	0.21	0.26	0.28
$Er(NO_3)_3$		0.00	0.00	0.00	0.00	0.10	0.18	0.20	0.23	0.00	0.03	0.01	0.15	0.23	0.25	0.26
$Tm(NO_3)_3$		0.00	0.00	0.00	0.04	0.07	0.08	0.14	0.12		0.08	0.09	0.19	0.26	0.29	0.27
$Th(NO_3)_4$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.09	0.18	0.21	0.31	0.32
GeO_2	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.08	0.09	0.09	0.12	0.15	0.18
$SnCl_2$	0.41				0.30		0.33		0.31	0.39	0.55		0.67	0.73		0.78
$AsCl_3$	0.41	0.40	0.42	0.40	0.41	0.41	0.41	0.46	0.40	0.41	0.40	0.45	0.40	0.44	0.48	0.44
$SbCl_3$	0.41	0.24	0.23	0.26	0.25	0.33	0.33	0.30	0.26	0.37						
$Bi(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.10	0.16	0.23	0.25	0.26
$Cr(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.03	0.15	0.22	0.46	0.30
$UO_2(NO_3)_2$	0.02	0.04	0.03	0.03	0.03	0.24	0.28	0.23	0.25	0.04	0.46 t	0.56 t	0.64	0.65	0.65	0.82
Na_2SeO_3	0.00	0.00	0.00	0.00	0.00	0.01	0.03	0.03	0.06	0.00	0.42	0.49	0.46	0.54	0.51	0.48
$Mn(NO_3)_2$	0.00	0.01	0.00	0.00	0.05	0.20	0.23	0.28	0.28	0.00	0.12	0.12	0.16	0.23	0.30	0.32
NH_4TcO_4		0.07	0.07	0.07	0.04	0.11	0.15	0.15	0.20	0.08	0.35	0.33	0.35	0.36	0.27	0.29
$KReO_4$	0.01	0.04	0.04	0.03	0.04	0.11	0.16	0.19	0.21	0.07	0.34	0.36	0.42	0.39	0.39	0.39
NaI	0.02	0.01	0.01	0.01	0.03	0.03	0.06		0.09	0.07	0.30	0.28	0.33	0.50	0.50	0.37
$Fe(NO_3)_3$	0.00	0.00	0.00	0.00	0.00	0.06 t	0.10 t	0.06 t	0.08 t	0.00	0.37 t	0.42 t	0.45 t	0.54 t	0.56 t	0.57 t
$Co(NO_3)_2$	0.00	0.01	0.01	0.01	0.01	0.14	0.23	0.28	0.31	0.00	0.16	0.20	0.21	0.29	0.40	0.33
$Ni(NO_3)_2$	0.00	0.01	0.02	0.01	0.02	0.17	0.26	0.31	0.39	0.00	0.12	0.15	0.20	0.26	0.28	0.31
$PdCl_2$	0.39	0.23 t	0.21 t	0.24 t	0.19 t	0.18 t	0.23	0.27	0.29	0.03	0.46	0.43	0.52	0.50	0.49	0.49
H_2PtCl_4	0.00	0.00	0.00	0.00	0.00	0.12	0.19	0.25	0.26	0.10	0.43	0.44	0.53	0.47	0.54	0.57

absence of nitric acid, one can see that, in the presence of acid, the values are either the same or higher.

Cations showing equal R_F values in both systems are those forming water- and/or alcohol-insoluble compounds ($R_F = 0$), and those forming relatively stable undissociated thiocyanate complexes (whose extractability is not influenced by the acid present in the eluant), such as copper, cobalt, nickel, manganese, cadmium, yttrium and the rare earths. It must here be recalled that all the cations belonging to this group, *i.e.* those that have equal R_F values in both systems, showed R_F values of zero or approximately zero in the previously studied systems containing isoamyl alcohol and various concentrations of nitric acid[4].

The effect of the nitric acid can possibly be explained either by the inhibition of hydrolysis and the formation of more extractable acid thiocyanate complexes, as in the case of iron, zinc, mercury and uranyl, or by the inhibition of hydrolysis preventing the formation of insoluble compounds of more or less amphoteric covalent species, such as those of aluminium, beryllium, indium and gallium.*

Systems with methyl isobutyl ketone

It was our intention to carry out experiments with methyl isobutyl ketone parallel to those with isoamyl alcohol. However, the preliminary chromatographic experiments showed that, when the eluant used was methyl isobutyl ketone saturated with ammonium thiocyanate (without nitric acid), practically all the cations listed in the experimental part gave R_F values of zero. In the presence of nitric acid, however, a number of cations showed R_F values other than zero. In view of the negative chromatographic results obtained in the system MIBK–NH_4CNS–H_2O, only the second system MIBK–NH_4CNS–HNO_3–H_2O was thoroughly investigated. The distribution of the two solutes, the thiocyanate and the acid, between water and the ketone, is presented in Table IV. The amounts of thiocyanate extracted by the ketone are very small, and its distribution coefficient decreases with increasing concentrations of thiocyanate in the initial aqueous solution, but at high initial thiocyanate concentrations the distribution coefficient remains practically constant. This low solubility of the thiocyanate in the ketone is further indicated by the unchanged volume ratios of organic and aqueous layers after equilibration, as shown in column 8.

Comparing the numerical values of the normalities of the thiocyanate with those of the acid in the organic phase (columns 6 and 7), it can be seen that equinormal amounts of both are extracted by the ketone, using concentrations up to approximately 2 M of thiocyanate in the initial aqueous solution. A further indication of the composition of the species extracted by MIBK can be derived from the results presented in Fig. 1. The slope of the straight line obtained by plotting log $\alpha_{[H^+]}$ *versus* the logarithm of the thiocyanate concentrations in the initial aqueous solution, is equal to one, which is similar to the results obtained with the isoamyl alcoholic

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* Note added in proof: The authors are much indebted to Dr. LEDERER, Editor of this Journal, for kindly suggesting the dismutation of $Fe(CNS)_3$ to Fe^{++} and $(CNS)_2$ as a plausible explanation for the tailing of iron on the chromatograms.

TABLE IV

THE DISTRIBUTION OF AMMONIUM THIOCYANATE AND NITRIC ACID BETWEEN WATER AND METHYL ISOBUTYL KETONE

No. of soln.	*Normality in the initial solution*		*Normality in the aqueous phase*		*Normality in the organic phase*		V_o/V_w	*Distribution coefficients of*			
								HNO_3		*NH_4CNS*	
	HNO_3	*NH_4CNS*	*HNO_3*	*NH_4CNS*	*HNO_3*	*NH_4CNS*		*a*	*μ*	*a*	*μ*
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
1	0.097	0.00	0.095	0.00	0.002*	0.00	1.00			—	—
2	0.097	0.09	0.067	0.063	0.030*	0.027*	1.00	0.45	0.45	0.43	0.43
3	0.097	0.18	0.054	0.135	0.043	0.040	1.00	0.79	0.79	0.30	0.30
4	0.097	0.45	0.032	0.39	0.065*	0.060	1.00	2.04	2.04	0.15	0.15
5	0.097	0.92	0.017	0.83	0.081	0.084	1.00	4.77	4.77	0.10	0.10
6	0.097	1.84	0.009	1.75	0.088	0.091	1.00	9.78	9.78	0.052	0.052
7	0.097	2.76	0.005	2.58	0.093	0.10	1.00	18.5	18.5	0.039	0.039
8	0.097	3.68	0.003	3.48	0.094*	0.12	1.00	31.4	31.4	0.034	0.034
9	0.097	5.52	0.0012	5.18	0.096*	0.25	1.00	79.8	79.8	0.048	0.048

* Calculated from the differences.

systems described above. This slope indicates that each hydrogen ion is attached to one thiocyanate ion. A change in the slope occurs when the initial thiocyanate concentration exceeds 2 *M*, coincidental with non-equinormal amounts of hydrogen and thiocyanate ions extracted by the ketone.

Chromatograms of the ions listed in the experimental part were run in methyl isobutyl ketone saturated with aqueous ammonium thiocyanate solutions of varying concentrations (0.1–5.5 *M*). With the exception of covalent chloride compounds, compounds of arsenic, antimony and tin, all the cations gave zero R_F values. The high extractability of these three covalent chlorides by methyl isobutyl ketone has already been observed[1, 2]. No thiocyanate compounds, either complexes or salt-like, are known for these cations. On the other hand, the addition of nitric acid to the thiocyanate solution resulted in R_F values other than zero, as shown in Table V. Almost all the cations moved on the paper with tailing. This tailing is generally

TABLE V

R_F VALUES OF IONS IN METHYL ISOBUTYL KETONE EQUILIBRATED WITH AQUEOUS SOLUTIONS CONTAINING NITRIC ACID AND AMMONIUM THIOCYANATE OF VARIOUS CONCENTRATIONS

M HNO_3	*0.097*								
M NH_4CNS	*0*	*0.09*	*0.18*	*0.45*	*0.92*	*1.84*	*2.76*	*3.68*	*5.52*
$Be(NO_3)_2$	0.00	0.18 t	0.25 t	0.24 t	0.28 t	0.26 t	0.30 t	0.36 t	0.42 t
$Zn(NO_3)_2$	0.00	0.32 t	0.41 t	0.40 t	0.45 t	0.46 t	0.44 t	0.48 t	0.50 t
$In(NO_3)_3$	0.00	0.35	0.39	0.48		0.56 t	0.55 t	0.60 t	0.57 t
$SbCl_3$	0.17	0.15	0.14	0.19	0.21	0.19	0.16	0.15	0.15
$UO_2(NO_3)_2$	0.08	0.26 t	0.31 t	0.31 t	0.36 t	0.40 t	0.37 t	0.40 t	0.43 t
$Mn(NO_3)_2$	0.00	0.25 t	0.28 t	0.32 t	0.41 t	0.35 t	0.37 t	0.38 t	0.37 t
$Fe(NO_3)_3$	0.00	0.22 t	0.29 t	0.33 t	0.36 t	0.37 t	0.13 t	0.12 t	0.12 t
$Co(NO_3)_2$	0.00	0.05 t	0.10 t	0.08 t	0.12 t	0.30 t			0.37 t
$PdCl_2$	0.00	0.11 t	0.08 t	0.10 t	0.08 t	0.08 t	0.10 t	0.13 t	0.08 t

caused by the very slow rate of hydrolysis of the compounds in question. Uncharged species, the only ones that could be extracted by a non-polar organic solvent (such as methyl isobutyl ketone), were probably formed as products of a slow and irreversible hydrolysis of the metal species. The usefulness of chromatographic separations in the above systems is therefore very limited. The effect of increasing the thiocyanate concentration could only be observed in the cases of cobalt and beryllium. The phenomenon is clearly a consequence of the limited solubility of the complexant itself in the organic phase, as pointed out above.

Only the presence of acid in the eluant affected the mobility of the listed cations. The influence of the acid must be similar to its influence in the systems with isoamyl alcohol, *i.e.* promoting the formation of uncharged thiocyanate complexes as in the case of ferric iron, cobalt, manganese and uranium, or inhibiting hydrolysis as in the case of amphoteric beryllium and indium. The R_F values of covalent antimony chloride are not influenced by the presence of acid.

Systems with di-isopropyl ether

Since one of the main aims of this research was to make a comparative study of the chromatographic behaviour of metallic nitrates in systems containing an alcohol, a ketone or an ether, we intended to carry out experiments parallel to those given above, using di-isopropyl ether as the representative of the ethers. Chromatographic experiments with systems containing various concentrations of ammonium thiocyanate with and without 0.1 M nitric acid, showed R_F values of zero irrespective of the thiocyanate concentration and the presence of nitric acid. The only exceptions were the cations, such as antimony, which owing to their covalent bonds, are readily soluble in ether. In view of the negative chromatographic results we did not find it worthwhile to undertake a comprehensive study of the distribution of the thiocyanate and the acid between water and the ether.

CONCLUSION

Ammonium thiocyanate is readily soluble in isoamyl alcohol, only very sparingly soluble in methyl isobutyl ketone and probably completely insoluble in di-isopropyl ether. Thiocyanic acid is easily extractable by the first two solvents and probably inextractable by the ether. The extractability of ammonium thiocyanate from an aqueous solution by an organic solvent seems to be connected with an appreciable swelling of the organic phase. This means that the extracted ammonium thiocyanate molecule must be readily hydrated. On the other hand, the extraction of thiocyanic acid does not seem to cause a swelling of the organic phase, which suggests that the extracted species is unhydrated.

The moving of cationic species on the paper depends mainly on the charge of the species and on the polarity of the organic solvent: non-polar organic solvents can extract only uncharged species. Since the thiocyanate metallic species formed during these chromatographic experiments were inextractable by the three non-polar organic solvents, saturated with aqueous solutions of ammonium thiocyanate, it follows that these species must be either cationic or anionic but not uncharged. Increased water content in the non-polar isoamyl alcohol (dielectric constant 5.7) increases the polarity of the water–alcohol mixture and enables even the charged thiocyanate species to move on the paper.

The presence of free thiocyanic acid in the organic solvent, apart from its effect of inhibiting hydrolysis, promotes the formation of less dissociated thiocyanate complex species, according to the reaction:

$$n\mathrm{NH_4}^+ + [\mathrm{M(CNS)}_x]^{-n} + n\mathrm{HNO_3} \rightarrow \mathrm{H}_n\,[\mathrm{M(CNS)}_x] + n\mathrm{NH_4NO_3}$$

The hydrogen ion here plays the role of the cation. The acid–complex species are more extractable by non-polar organic solvents than the corresponding ammonium salts, and therefore higher R_F values were obtained in systems containing nitric acid.

SUMMARY

Data are presented for the partition of nitric acid and/or ammonium thiocyanate between (1) water and isoamyl alcohol, (2) water and methyl isobutyl ketone. The R_F values of about sixty ions have been measured in isoamyl alcohol, methyl isobutyl and di-isopropyl ether, saturated with aqueous solutions containing varying amounts of ammonium thiocyanate and nitric acid. The effects of the solutes on the extractability of metallic species from aqueous solution by alcohol, ketone and ether, have been discussed and compared.

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THE PAPER CHROMATOGRAPHIC SEPARATION OF COBALT(II) CHLORIDE AND SOME AMMINE AND ETHYLENEDIAMINE COMPLEXES OF COBALT(III)

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INTRODUCTION

In the paper-chromatographic separation of metal–ammine complexes, the metal can either be converted to the complex by using an ammoniacal solvent system, or it can be applied to the paper as a prepared complex.

ERDEM AND ERLENMEYER[1] have studied the behaviour of cadmium acetate in ammoniacal solvents, and interpret the triple spotting of cadmium as indicating the separation of different ammine complexes. A few separations of trivalent cobalt complexes have been described. YAMAMOTO *et al.*[2] and LEDERER[3] have investigated some ammine complexes; STEFANOVIĆ AND JANJIĆ[4] have separated the geometric isomers of three ethylenediamine complexes.

Since the work described here was completed, SINGH AND DEY[5] have obtained separations of the ammines of copper, silver, cadmium, nickel and cobalt, using 50% ethanol as eluting solvent. Further, AGRINIER[6], in a study of the application of paper chromatography to the determination of certain elements in minerals, has separated silver, lead, selenium, zinc and copper by elution with concentrated ammonia.

It has now been found possible to separate copper, cobalt and nickel, and zinc from cadmium using a selected ternary solvent system of methanol–water–ammonia, which does not give double-spotting with cadmium. The behaviour of 14 ammine and ethylenediamine complexes in: acetone–aqueous hydrochloric acid, methanol–aqueous hydrochloric acid, and ether–methanol–aqueous hydrochloric acid has been investigated in some detail, and a number of new separations are described. Brief mention of some of this work has been made elsewhere[7].

EXPERIMENTAL

Preparation of the solutions

0.1 *N* solutions of copper, cobalt, nickel, zinc and cadmium acetates were used for spotting the chromatograms described in Section A. The complexes $Co(en)_3Cl_3$*, $[Co(en)_2Cl_2]Cl$, and $[Co(en)_2(H_2O)_2]Cl_3$ were prepared as follows:

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* en = ethylenediamine.

(a) Tris(ethylenediamine) cobalt(III) chloride[8], $Co(en)_3Cl_3$. This was prepared by the aerial oxidation (3 hours) of aqueous cobaltous chloride (24 g:75 ml H_2O) in the presence of 30% ethylenediamine (61 g) and 6 *N* HCl (17 ml). After evaporation to a small volume on a steam bath, the complex crystallized in orange-yellow needles, easily soluble in water.

(b) trans-Dichlorobis(ethylenediamine) cobalt(III) chloride[9], $[Co(en)_2Cl_2]Cl$. Prolonged aerial oxidation (approx. 10–12 h) of aqueous cobaltous chloride (160 g:500 ml) and 10% ethylenediamine (600 g) was carried out. Excess concentrated HCl (350 ml) was added followed by evaporation to approx. 750 ml when the bright green plate-like crystals of the hydrochloride of the base were deposited.

(c) trans-Diaquobis(ethylenediamine) cobalt(III) chloride, $[Co(en)_2(H_2O)_2]Cl_3$. The green *trans*-isomer from (b) was converted to the red *cis*-isomer by evaporation in aqueous solution. This complex (2 g) was converted to the *cis*-dibromo-complex by cautious warming with NH_3 (10 ml; $d = 0.880$) followed by trituration with solid NaBr. The dibromo-complex crystallized in red needles, and was purified from a little H_2O by reprecipitation with NaBr[10]. It was converted to the *cis*-diaquo-complex by cautious warming with the equivalent amount of conc. HCl[11].

The aqueous solutions each contained 10 μg cation/μl. The other cobalt(III) solutions were of a similar concentration (except where stated otherwise). The preparation of these complexes is now described:

(d) Hexammino-cobalt(III) chloride[12], $Co(NH_3)_6Cl_3$. A mixture of cobaltous chloride (18 g) dissolved in ammonium chloride (12 g in 25 ml H_2O) was mixed with NH_3 (40 ml; $d = 0.880$) and oxidised in the cold with "20 volume" hydrogen peroxide (35 ml). On warming to 60° the required complex was formed in golden-brown crystals.

(e) Ammonium tetranitro-diammino-cobaltate. Erdmann's salt, $NH_4[Co(NH_3)_2(NO_2)_4]$. Ammonium chloride (20 g) and sodium nitrite (27 g) were dissolved in H_2O (150 ml). After filtration, the filtrate was mixed with cobaltous chloride (18 g in 50 ml H_2O) and 20% aqueous ammonia (5 ml) and aerial oxidation carried out for 1½ h. On spontaneous evaporation, brown crystals of the required complex were formed.

(f) Trinitro-triammino-cobalt(III)[12], $Co(NH_3)_3(NO_2)_3$. Cobalt carbonate (7 g) was dissolved in a mixture of water (40 ml) and glacial acetic acid (10 ml), and added to a solution of sodium nitrite (15 g) in NH_3 (70 ml; $d = 0.880$). The mixture was oxidised in the cold with "20 volume" hydrogen peroxide (20 ml). On warming the mustard-yellow complex was produced, and was recrystallized from very dilute acetic acid.

(g) cis-Dinitrotetrammine cobalt(III) chloride, 1:2$[Co(NH_3)_4(NO_2)_2]Cl$. Carbonato-tetrammine cobaltic nitrate (5 g) was dissolved in water (50 ml) and conc. nitric acid (3 ml), treated with sodium nitrite (10 g), warmed for 10 min, cooled and acidified with 6 *N* nitric acid (65 ml). After standing for 24 h the precipitate of mixed acid and neutral *flavo*nitrate was removed and recrystallized from *very* dilute acetic acid. It was converted to the required chloride form by dissolution in water (1 g in 30 ml)

followed by treatment with ammonium chloride (2 g), the complex being precipitated by the gradual addition of methylated spirit (100 ml).

(h) trans-Dinitrotetrammine cobalt(III) chloride, $1{:}6[\mathrm{Co(NH_3)_4(NO_2)_2}]\mathrm{Cl}$. To a cold filtered solution of ammonium chloride (4 g) and sodium nitrite (5.5 g) in water (30 ml) was added 2 *N* ammonia (10 ml) and cobaltous chloride (3.5 g in 10 ml H_2O). Aerial oxidation was carried out for 4 h, and after standing for 24 h the required complex was filtered off. It was purified by dissolution in very dilute acetic acid and reprecipitation with concentrated ammonium chloride solution.

(i) Chloro-pentammine cobalt(III) chloride, $[\mathrm{Co(NH_3)_5Cl}]\mathrm{Cl_2}$. Conc. HCl (4.5 ml) was added to a solution of carbonatotetrammine cobaltic nitrate (3 g in 40 ml H_2O). After removal of all CO_2 the solution was made slightly ammoniacal, and an excess of NH_3 (5 ml; $d = 0.880$) added. The solution was heated for ¾ h, cooled, and conc. HCl (50 ml) added. After further warming for 1 h, the solution was allowed to cool, when it deposited violet-red crystals of the required complex.

(j) Sodium cobaltinitrite[12], $\mathrm{Na_3[Co(NO}$ [illegible]]. Cobaltous nitrate (10 g) was dissolved in a warm solution of sodium nitrite (30 g [illegible] 0 ml H_2O), and 50% acetic acid (10 ml) gradually added with good stirring. After aerial oxidation for ½ h, ethanol (50 ml) was added at 0° with good stirring; the orange-coloured crystals were filtered and washed with ethanol.

(k) Potassium trioxalato-cobaltate[12], $\mathrm{K_3[Co(Ox)_3]}{:}3\tfrac{1}{2}\,\mathrm{H_2O}$. Oxalic acid dihydrate (12.6 g), potassium oxalate (36.8 g), and cobalt carbonate (12 g) were dissolved in boiling water (250 ml). After cooling the solution to 30°, finely divided lead dioxide (30 g) was slowly added with good stirring, followed by a uniform mixture of glacial acetic acid (12.5 ml) and water (12.5 ml), the addition of which should take not less than ½ h. After spontaneous oxidation (with stirring) for 1 h, and removal by filtration of excess dioxide, the complex was precipitated as dark green crystals by slow addition of ethanol (250 ml).

(l) Ammonium diammino-dinitro-oxalato-cobaltate, $\mathrm{NH_4[Co(NH_3)_2(NO_2)_2Ox]}$. Erdmann's salt (5 g) was dissolved in warm water (25 ml) and oxalic acid (2.5 g in 12.5 ml H_2O) added at 50°. After standing for 24 h, the crystalline precipitate was filtered off (A). To the filtrate (B) was added 2.5 g ammonium chloride as saturated solution; this precipitated the complex $[\mathrm{Co_4(NO_2)_6(C_2O_4)_3\ (NH_3)_6}]\mathrm{NH_4{\cdot}6H_2O}$, and the solution after filtration of this complex was found to contain the required ammonium salt of $[\mathrm{Co(NH_3)_2(NO_2)_2Ox}]^-$. This complex was also found in the filtrate obtained by dissolving the crystals (A) in water, and precipitating the complex $[\mathrm{Co_4(NO_2)_6(Ox)_3(NH_3)_6}]\mathrm{NH_4}$ as above.

Descending elution technique

Section A. The all-glass apparatus[13] consisted of two troughs (24 cm long and 2.5 cm in diameter) supported by a glass stand contained in a tank (23 cm × 28 cm × 52 cm deep) covered with a ground-glass plate drilled with two holes located one over each of the two troughs. The purpose of the holes was to permit solvent to be admitted into the trough without removing the glass cover-plate, and so disturbing the equilibration of the tank.

Sheets of Whatman No. 1 paper 10 cm × 40 cm were used for the chromatograms, with the starting line marked 8 cm from the upper narrow edge. Two creases, 4 cm and 6 cm above the starting line produced a V-shaped fold which could then be placed in the glass trough. The paper strip was held in position with a piece of glass rod fitted with a central side arm to allow easy removal after elution. 0.5 cm spots of the solutions were placed 2 cm apart on the starting line and allowed to dry. The paper was placed in a trough in the tank; a second trough was filled with solvent and equilibration of tank and paper allowed to proceed overnight. A quantity of the eluting solvent was placed in the empty trough, and elution allowed to proceed for 2–3 h, the temperature being maintained at 18° ± 2°. The length of run was 25–40 cm. The chromatogram was then removed, dried and sprayed as follows.

Rubeanic acid[14] was used to detect the copper, cobalt and nickel, and 8-hydroxyquinoline[15] revealed the positions of the zinc and cadmium.

Section C. The apparatus was smaller than that used in Section A, the tank being 10 cm × 16.5 cm × 31 cm deep. Equilibration of the tank was carried out for 24 h, and of the paper for 45 min. The length of run was 20 cm on Whatman No. 1 paper. Other conditions were similar to those above.

Ascending elution technique

Section B. The apparatus consisted of a gas-jar (30 cm high × 8 cm diameter), the top of which was accurately ground flat and greased with vaseline. 100 ml of solvent was introduced into the jar, which was then sealed by two flat glass plates, (14 cm × 5 cm), ground flat on the 14 cm sides. A cover plate (14 cm × 9 cm) was placed over these two slides, and held in position by two rubber bands. Before elution was commenced, the apparatus was equilibrated for 1 h. The cover plate was then removed, the slides were gently displaced sideways and the paper strip inserted until the lower edge just touched the solvent surface. The protruding upper end of the strip was folded to lie flat along the surface of the slides, which were then pushed tightly together and again held in position by the cover plate and rubber bands.

Whatman No. 1 paper, cut perpendicularly to the machine direction into strips 6 × 30 cm, was used for the chromatograms. The length of run was 15.0 ± 0.2 cm at 18° ± 2°. The size of the spots was standardised at 1.5 μl using a calibrated capillary dropper[17].

Section A. The separation of copper, cobalt and nickel, and zinc from cadmium

Preliminary experiments showed that as the ammonia concentration increased above 15 ml, the spot compactness improved. The solvent mixtures given in Table I were next investigated.

At 15 ml water (interpolating from the results at 10 and 20 ml water where necessary) an increase in the volume of conc. ammonia from 10 to 40 ml resulted in

TABLE I

Solvent number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Volume methanol	70	65	55	60	55	45	60	55	50	40
Volume water	5	10	20	10	15	25	0	5	10	20
Volume ammonia, $d = 0.88$	25	25	25	30	30	30	40	40	40	40

the elevation of all R_F values. Copper and nickel gradually "pulled away" from the cobalt (which never completely left the starting line) so that at 30 ml conc. ammonia a good separation of copper, cobalt and nickel was achieved. The blue colour of the copper spot during elution showed that it moved as the ammine. The separation of zinc and cadmium was also at its best with this solvent mixture. Above 30 ml ammonia, the spots became distended and the separations inferior.

Variations in the water concentration, with the conc. ammonia maintained at 30 ml, confirmed that the optimum solvent composition for the separations Cu–Co–Ni and Zn–Cd, was: 55 ml methanol + 15 ml water + 30 ml conc. ammonia. R_F values were: Co 0–0.3 (bulk at very low R_F), Cu 0.35–0.50, Ni 0.55–0.70, Zn 0.34–0.53, and Cd 0.70–0.79.

The forward tailing of cobalt was presumably due to the atmospheric oxidation of cobalt(II) at the starting line to cobalt(III), which then moves to R_F 0.30. In many of the solvents investigated, cadmium produced two spots on the chromatogram. This was attributed *either* to the effect of sharp solvent composition gradients along the paper (*e.g.* a water front) *or* to the separation of two ammines of cadmium (*cf.* ref[1]). The former is considered to be the more likely, and compares with the double spot phenomenon observed for certain organic molecules[18].

A number of ternary mixtures taken from the solvent systems: $CH_3(CH_2)_2OH$–H_2O–NH_3, $(CH_3)_2CHOH$–H_2O–NH_3, and $(CH_3)_2CO$–H_2O–NH_3 showed no appreciable improvement on the results obtained with the methanol solvent just described. However, $(CH_3)_2CHOH$–H_2O–conc. ammonia (40:30:30, v/v) produced an excellent separation of copper (R_F 0.3–0.6) and nickel (R_F 0.7–0.8).

Section B. The separation of cobalt(II) chloride from some cobalt(III) ethylenediamine complexes

The solvent systems $(CH_3)_2CO$–H_2O–HCl and CH_3OH–H_2O–HCl were investigated over wide ranges of composition (Table II) for the separation of $CoCl_2$ from $Co(en)_3Cl_3$, $[Co(en)_2Cl_2]Cl$, and $[Co(en)_2(H_2O)_2]Cl_3$.

The complexes always remained very close together on the chromatogram with no separation of the adjacent extremities of the spots. In the acetone solvents maximum separation of $CoCl_2$ from the nearest complex was obtained with $(CH_3)_2Co$–H_2O–conc. HCl (sp. gr. = 1.18), 88:6:6 (v/v), with an R_F separation of 0.15 of the adjacent extremities of the spots of $CoCl_2$ and $Co(en)_3Cl_3$. The R_F values of the centres of gravity of the spots were: $CoCl_2$ = 0.32, complexes = 0.05–0.06.

TABLE II

Solvent numbers	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*
	22	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*
Volume methanol* or acetone**	92	90	88	90	88	86	84	86	84	82	80
Volume water	6	6	6	8	8	8	8	10	10	10	10
Volume conc. HCl	2	4	6	2	4	6	8	4	6	8	10

* Solvents 11–21.
** Solvents 22–32.

The system methanol–aqueous hydrochloric acid produced greater centre of gravity R_F separation of $CoCl_2$ from the complexes, but unfortunately the spot size was greater than with the acetone solvents. At the optimum solvent composition, methanol–water–conc. HCl, 92:6:2(v/v), the R_F values were: $CoCl_2$ = 0.70, complexes = 0.18–0.23.

The separation of $Co(en)_3Cl_3$ from $[Co(en)_2Cl_2]Cl$ and $[Co(en)_2(H_2O)_2]Cl_3$

With solvents 33–37, all the solutes moved very little or not at all. With solvents 38–41, the spots were very compact and moved some way from the starting line. However, with the two remaining solvents (42 and 43) the spots became much larger, resulting in considerable overlapping.

TABLE III

Solvent number	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*	*43*
Volume ether	81.3	78.9	78.1	77.6	76.7	55.8	53.0	52.0	50.9	49.2	47.5
Volume methanol	16.3	16.8	15.6	15.5	15.3	34.5	31.8	31.2	30.6	29.5	28.5
Volume water	0.0	3.2	4.8	6.1	8.0	0.0	5.4	7.8	10.2	17.3	24.0
Volume conc. HCl	2.4	2.1	1.5	0.8	0.0	10.7	9.9	9.1	8.3	4.0	0.0

Without the ether, solvents 38–41 gave a slight R_F separation at a high R_F value, but with some overlapping. The addition of ether resulted in a "pulling back" of the spots to lower R_F values, but the *distance* of separation did not decrease commensurately with the fall in R_F. Consequently, solvents 38–41 were re-investigated by descending elution using the same procedure as adopted in Section C. It was found that the increase in the length of the run more than offset the effect of the added ether. In fact a separation of $Co(en)_3Cl_3$ from $[Co(en)_2Cl_2]Cl$ and $[Co(en)_2(H_2O)_2]Cl_3$ was obtained, typical R_F values being those for solvent number 40:

$CoCl_2$ = 0.16, $Co(en)_3Cl_3$ = 0.16, $[Co(en)_2Cl_2]Cl$ = 0.09, $[Co(en)_2(H_2O)_2]Cl_3$ = 0.095. The acid front in this solvent was at R_F 0.70.

There was some slight reduction of the dichloro-complex to Co(II), but this was so slight as to be hardly detectable. Further, there was a very faint spot at R_F = 0.12 in the $[Co(en)_2(H_2O)_2]Cl_3$ "lane" which did not correspond to any of the other solutes on the chromatogram. It appears that this is due to a product of an equilibrium reaction on the chromatogram, possibly $[Co(en)_2(H_2O)Cl]Cl_2$. This could quite easily be produced by the equilibrium:

$$[Co(en)_2(H_2O)_2]Cl_3 \rightleftharpoons [Co(en)_2(H_2O)Cl]Cl_2 + H_2O$$

particularly in the presence of an HCl-containing solvent and reactive cellulose.

Section C. The separation of some cobalt(III) complexes

It has already been reported in a preliminary communication[7] that the solvent system: ether–methanol–water–conc. HCl, 50:30:20:2 (v/v), is extremely valuable for the separation of some ammine complexes of cobalt(III). Full details are now given in Table IV and the appended remarks.

TABLE IV

	Solute	*R_F values*		*Remarks*
		mean	*tailing limits*	
1	$CoCl_2$	0.43	(0.35–0.49)	Compact spot
2	$NH_4[Co(NH_3)_2(NO_2)_2Ox]$	0.74	(0.73–0.75)	Slight tailing to R_F 0.35*
3	$NH_4[Co(NH_3)_2(NO_2)_4]$	0.31	(0.28–0.33)	Some tailing to R_F 0
4	$Co(NH_3)_3(NO_2)_3$	0.08	(0.05–0.11) and	
		0.34	(0.30–0.38)	
5	*cis*-$[Co(NH_3)_4(NO_2)_2]Cl$	0.23	(0.18–0.27)	Faint tailing to R_F 0.10
6	*trans*-$[Co(NH_3)_4(NO_2)_2]Cl$	0.74	(0.73–0.75)	Slight tailing to R_F 0**
7	$[Co(NH_3)_5Cl]Cl_2$	0.14	(0.11–0.17)***	
8	$Co(NH_3)_6Cl_3$	0†, and		Main spot at R_F 0, but a little at 0.15
		0.15	(0.11–0.20)	
9	$Na_3Co(NO_2)_6$	0.43	(0.35–0.49)	Some tailing to 0.70. Main spot = Co(II) (due to rapid conversion of the complex during elution)
10	K_3CoOx_3	0.66	(0.62–0.70)	Some tailing to 0.35 (due to decomposition)
11	*cis*- or *trans*-$[Co(en)_2Cl_2]Cl$	0.27	(0.19–0.33)	Compact spot

The R_F value of the acid front was 0.75. Ox = $(COO)_2^{-2}$. en = ethylenediamine.

* The tailing back to R_F 0.35 indicates decomposition to Co(II) during elution.

** There was some tailing between spots, and back to R_F 0.The double-spotting suggests either a *cis–trans* separation, or a separation of the nitro- and nitrato-isomers.

*** An intensification of the tailing at R_F 0.34 suggests decomposition to one of the components present in the spot of $Co(NH_3)_3(NO_2)_3$. The original solution contained solid chloro-pentammino-cobaltic chloride (purpureo cobaltic chloride) dissolved in the eluting solvent.

† The spot at R_F 0 was very probably due to hydrolysis at the starting line.

SUMMARY

By use of an eluting solvent containing ammonia dissolved in aqueous methanol, it has been found possible to separate by paper chromatography copper, cobalt and nickel, and zinc from cadmium.

Certain new separations of a variety of ammine and ethylenediamine complexes of cobalt(III) are described, the eluting solvents used being ternary and quaternary mixtures of ether, methanol, water and concentrated hydrochloric acid.

References p. 379.

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ÜBER PAPIERIONOPHORESE BEI SPANNUNGSGEFÄLLEN VON 50 V/cm

IV. ÜBER DIE PAPIERIONOPHORESE VON ABBAUSÄUREN (DICARBONSÄUREN)*

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Eine der wichtigsten chemischen Methoden zur Strukturaufklärung organischer Stoffe ist der oxydative Abbau. Das bei dieser Methode anfallende Reaktionsprodukt enthält meistens ein komplexes Gemisch organischer Säuren, dessen Auftrennung mit klassischen chemischen Methoden oft nicht leicht ist.

Verteilungschromatographische Verfahren führen nur teilweise zum Ziel. Wenn Hochvoltelektrophorese in der richtigen Weise angewandt wird, erhält man schnell Ergebnisse über Art und Anzahl der entstandenen Säuren. In erster Linie werden nicht flüchtige, zwei oder mehrere Carboxylgruppen enthaltende Säuren erfasst.

Bei den chromatographischen Verfahren[1] ist der Zusammenhang zwischen der chemischen Konstitution der Säure und ihrer Lage im Chromatogramm nicht immer klar erkennbar. Dagegen liefert die Hochvoltelektrophorese, da mehrbasische organische Säuren vom Filtrierpapier fast nicht adsorbiert werden, einen deutlichen Zusammenhang zwischen der Dissoziationskonstante und der Wanderungsgeschwindigkeit[2,3]. Ferner kann der Einfluss der chemischen Konstitution auf die Dissoziationskonstante der Säure häufig abgeschätzt werden, daher ist die Identifizierung einer unbekannten Säure lediglich aus ihrer Lage im Elektropherogramm und durch Vergleich mit den in Tabelle I und Fig. 1 angegebenen Wanderungsgeschwindigkeiten wesentlich erleichtert.

Durch Vergleich einer unbekannten Dicarbonsäure mit den Vertretern der homologen, nicht verzweigten Dicarbonsäuren erhält man zunächst einen Hinweis auf die Zahl der Kohlenstoffatome zwischen den Carboxylgruppen. Aus der Tabelle kann man erkennen, dass verzweigte Dicarbonsäuren nur wenig langsamer als die entsprechenden nicht verzweigten, also auf alle Fälle viel schneller als das nächst höhere Homologe wandern. Säuren des Typs $HOOC{-}(CH_2)_n{-}CHR{-}COOH$ unterscheiden sich in ihrer Wanderungsgeschwindigkeit kaum von den Homologen der Hauptreihe. Durch den induktiven Effekt der α-ständigen Alkylgruppe wird ja nur die zweite Dissoziationskonstante (K_2) beeinflusst, während die bei den tabellierten pH-Werten die Wanderungsgeschwindigkeit bestimmende K_1 fast gleich der

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* III. Mitt. siehe Ref.[4].

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TABELLE I

RELATIVE GESCHWINDIGKEITEN (MALONSÄURE = 1.00) AUF SCHLEICHER-SCHÜLL 2043 B

Säure	*Essigsäure–Pyridin* 5.5 : 1.5 *pH* = 3.9	*Essigsäure–Pyridin* 5.5 : 2.5 *pH* = 4.15
Versuch bei 10° Kühlwassertemperatur		
Äthylmalonsäure	0.78	0.78
Adipinsäure	0.26	0.34
Bernsteinsäure	0.40	0.50
Diäthylmalonsäure	0.76	0.76
α,α'-Dimethyladipinsäure	0.24	0.32
Glutarsäure	0.32	0.42
n-Hexan-$\beta,\beta,\varepsilon,\varepsilon$-tetracarbonsäure	0.87	0.87
Malonsäure	1.00	1.00
Methylmalonsäure	0.83	0.83
Oxalsäure	1.10	1.10
Pimelinsäure	0.22	0.30
Benzoesäure	0.28	0.34
Isophthalsäure	0.47	0.55
Phthalsäure	0.59	0.59
Versuch bei 40°		
Benzoesäure	0.27	
Isophthalsäure	0.49	
Phthalsäure	0.61	

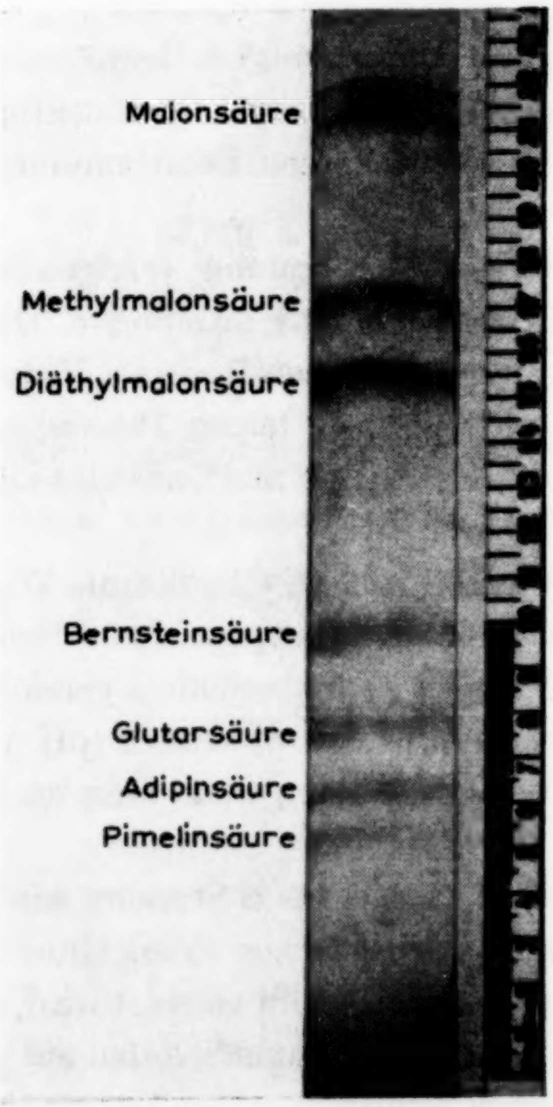
Malonsäure
Methylmalonsäure
Diäthylmalonsäure
Bernsteinsäure
Glutarsäure
Adipinsäure
Pimelinsäure

Fig. 1. 38 V/cm, 3.0 mA/cm Streifenbreite. Puffer: 5.5 ml Essigsäure, 2.5 ml Pyridin, auf 100 ml Wasser (pH 4.15).

unverzweigten Säure ist. So wandern die (nicht tabellierte) Methylbernsteinsäure und α-Methylglutarsäure praktisch genau so schnell wie Bernsteinsäure bzw. Glutarsäure. Eine Trennung erreicht man hier erst durch Einstellen des Puffers auf pH-Werte, bei denen die zweite Dissoziationskonstante wirksam wird. Für die Trennung höherer Säuren mit verschieden langen Seitenketten (Methyl- und Äthylmalonsäure lassen sich elektrophoretisch noch leicht trennen) sind papierchromatographische Verfahren vorzuziehen. Das gleiche gilt für Dicarbonsäuren, die nicht α-ständig verzweigt sind, etwa Methylglutarsäure und Glutarsäure.

Die genaue Identifizierung erfordert das Aufbringen entsprechender Vergleichssubstanzen. Die Wahl dieser Säuren wird durch die nun bekannte Zugehörigkeit zu einem Glied der homologen Reihe, die für oxydative Abbaureaktionen geltenden Gesetzmässigkeiten und eventuell durch eine teilweise Kenntnis der Struktur der abgebauten Substanz wesentlich erleichtert.

EXPERIMENTELLER TEIL

Die Apparatur und die Versuchstechnik wurde an anderer Stelle ausführlich beschrieben[4,5]. Verwendet wurde eine Apparatur vom Sandwich-Typ, die mit fliessendem Leitungswasser (10°) oder mit Hilfe eines Thermostaten auf die gewünschte Temperatur gebracht wurde.

Nach dem oxydativen Abbau—etwa mit $KMnO_4$—reichert man die Säuren durch Aufnehmen in Lauge und Ausäthern nach dem Ansäuren an. Unter Umständen kann auch eine Hochvakuumdestillation, bei der flüchtige Säuren abgetrennt und Polycarbonsäuren zu den übersichtlicheren Dicarbonsäuren decarboxyliert werden, zweckmässig sein.

Der Papierstreifen, in dem die Trennung erfolgen soll, wird mit dem Puffer getränkt und zwischen Filtrierpapier gut abgepresst. Das Gewicht des trockenen Streifens zum Gewicht des aufgenommenen Puffers soll etwa 1:1.0–1.2 betragen. Die Abbausäuren bringt man mittels eines feinen Pinsels oder einer Pipette bis zur Streifenmitte auf, die andere Hälfte der Streifenbreite ist für Vergleichssubstanzen reserviert.

Der pH-Wert des Puffers soll in der Nähe der pK-Werte der Säuren liegen. Die von uns am häufigsten verwendeten Puffergemische bestehen aus einem Gemisch von 5.5 ml Essigsäure und 1.5 ml nicht besonders gereinigtem Pyridin oder 5.5 ml Essigsäure und 2.5 ml Pyridin in je 100 ml Wasser (pH 3.9 bzw. 4.15). Das letztere Puffergemisch eignet sich besonders dann, wenn man im Bereich der längerkettigen Säuren operiert.

Nach einer Versuchsdauer von ½ bis 2 Stunden wird der Streifen bei 120° getrocknet und sodann mit einem Gemisch aus 10 mg Glucose, 5 mg Arabinose in 6 ml Äthanol–Butanol 1:1, das mit 0.5 ml Anilin versetzt wird, besprüht. Kurzes Erhitzen auf 140° lässt die Lage der Säuren als braune Streifen auf weissem Grund erscheinen. Ein anderes, auf dem gleichen Prinzip beruhendes Sprühmittel besteht aus einem Gemisch von 2 g Glucose, 2 ml Anilin in 50 ml Butanol–Äthanol–Wasser wie 6:2:2[6].

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Es empfiehlt sich, als Vergleichssubstanz immer Malonsäure mitlaufen zu lassen, da die Bestimmung der relativen Lage einfacher ist, als die der scheinbaren Ionenbeweglichkeit. Letztere lag bei Malonsäure und den für die Tabelle angegebenen Versuchsbedingungen bei $1.05 \cdot 10^{-4}$ cm^{2}V^{-1}sec^{-1} (10°) bzw. $1.6 \cdot 10^{-4}$ cm^{2}V^{-1}sec^{-1} (40°).

Sind die zu untersuchenden Säuren schwer löslich (z.B. Benzoesäure, Isophthalsäure), kann man bei erhöhter Temperatur arbeiten. Dabei muss man das Spannungsgefälle meist reduzieren, bei 40° etwa auf die Hälfte des bei 10° verwendeten.

Durch Verwendung breiter Streifen und mehrfaches Wiederholen des Versuches konnten bestimmte Abbausäuren der Bitterstoffe Lactucin und Laserpitin im mg-Masstab isoliert und durch Schmelzpunktbestimmungen sowie Papierchromatographie identifiziert werden[7].

ZUSAMMENFASSUNG

Im vorliegenden Aufsatz wird eine Methode zur Erkennung schwerflüchtiger Säuren, wie sie nach einem oxydativen Abbau entstehen, beschrieben.

Man kann mit Hilfe der Papierionophorese und unter Verwendung von Essigsäure-Pyridinpuffern komplizierte Gemische von Abbausäuren auftrennen.

SUMMARY

A method is described for the identification of acids that are difficultly volatile, such as those obtained by oxidative degradation. Complicated mixtures of these acids can be separated by means of paper ionophoresis, using acetic acid-pyridine buffers.

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PAPER CHROMATOGRAPHIC SEPARATION OF SOME ANTIOXIDANTS

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With the increased use of antioxidants today, both as food additives and in certain types of experimental work[1], there is a definite need for methods to separate and identify these compounds. Procedures for the separation of four food approved antioxidants have been described by MITCHELL[2]; however, a distinct separation of all components in one dimension was not achieved. In addition, ZIJP[3] and DEHORITY[4] have reported the use of paper chromatography in analyses for N,N′-diphenyl-*p*-phenylenediamine in rubber and milk, respectively. This paper describes methods for the separation and identification of eight antioxidants currently used either as food additives or in experimental work.

EXPERIMENTAL

Antioxidants

The antioxidants used in this study were as follows:

a. PG: *n*-propyl gallate
b. BHA (butylated hydroxyanisole): 2-*tert.*-butyl-4-hydroxyanisole
c. BHT (butylated hydroxytoluene): 2,6-di-*tert.*-butyl-*p*-cresol
d. NDGA (nordihydroguaiaretic acid): 4,4′-(2,3-dimethyltetramethylene)-dipyrocatechol
e. DPPD: N,N′-diphenyl-*p*-phenylenediamine
f. DTBH: 2,5-di-*tert.*-butylhydroquinone
g. Santoquin: 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline
h. α-Tocopherol.

Apparatus

Chromatography was carried out in the ascending direction, with the papers suspended from glass rods near the top of Pyrex cylinders (18 in. high and 6 in. diameter) covered with glass plates. The glass rods had a rubber "policeman" on each end and were wedged into the cylinder. The developing solvent was poured directly into the bottom of the cylinder.

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Papers

For the separations described below, partially acetylated paper and cottonseed oil coated paper were utilized. These papers were prepared for use as follows:

a. Acetylated paper: Whatman No. 1 filter paper was acetylated by a modified procedure developed according to the methods of KOSTIR AND SLAVIK (as reported by BALSTON AND TALBOT[5]) and MICHEEL (as reported by BLOCK, DURRUM AND ZWEIG[6]). Sheets of Whatman No. 1 paper, 16.25 × 2 inches each (the longer side perpendicular to the machine direction of the paper), were suspended in a liter graduate cylinder (containing a glass covered magnet) from a glass rod placed across the top, and held with stainless steel chromatography clips. The papers were then covered with the acetylating mixture, which contained 400 ml of acetic anhydride, 800 ml of benzene and 1.2 ml of conc. sulfuric acid. The top of the cylinder was sealed with aluminum foil, and placed on a magnetic stirrer which was used intermittently at slow speeds throughout the acetylation process. After acetylating for 16–20 hours, the papers were removed and air dried in a hood, washed for 15 minutes in cool running tap water followed by three 5 minute washings in distilled water, washed for 15 minutes in absolute methanol, and finally air dried in the hood. To prevent curling of the papers, it was found advantageous to attach a glass rod by means of chromatography clips to the bottom edge of the papers during this final drying.

b. Cottonseed oil coated paper: Whatman No. 1 filter paper was coated by dipping into a 10% solution of cottonseed oil[*] in benzene. The benzene was allowed to evaporate at room temperature in a hood after which the papers were ready for use.

Solvents

a. Acetone–water (60:40).
b. Acetone–ethyl acetate–water (30:10:60).
c. Absolute methanol–water (75:25).

Chromogenic agents

The developed chromatograms were sprayed with a 0.5% solution of phosphomolybdic acid in 95% ethanol and hung in an ammonium hydroxide atmosphere as described by MITCHELL[2]. With this procedure all of the antioxidants were visible as blue spots, except DPPD which gave a brown spot.

RESULTS AND DISCUSSION

Three methods were employed for chromatographic separation of the antioxidants. The first (I) utilized partially acetylated paper with 60% acetone as the developing solvent; the second (II) cottonseed oil paper and acetone–ethyl acetate–water as the solvent mixture; and the third (III) again used cottonseed oil paper, but the solvent was 75% methanol. The separations obtained are given in Table I. Each value represents the mean of at least five individual separations. R_{PG} values (distance moved by antioxidant divided by the distance moved by propyl gallate) were used in preference to R_F values since the former accounted for deviations observed between different batches of acetylated paper and different cottonseed oil solutions.

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[*] A commercially available winterized cottonseed oil (Wesson Oil), refined and distributed by the Wesson Oil and Snowdrift Sales Co., New Orleans, Louisiana, was used for this study.

Both I and III gave a distinct separation of six of the eight antioxidants. In I, BHA and Santoquin did not separate, while in III NDGA and PG were inseparable. In the report by MITCHELL[2], PG, BHA, BHT and NDGA were not completely separated by unidimensional chromatography, with PG and NDGA consistently overlapping in all the systems utilized. However, in the present study, a definite separation of PG and NDGA was accomplished by either method I or II.

TABLE I

MEAN R_{PG} VALUES OF VARIOUS ANTIOXIDANTS

Method I: Partially acetylated Whatman No. 1 paper using acetone–water (60:40) as a developing solvent. Method II: Cottonseed oil coated paper using acetone–ethyl acetate–water (30:10:60) as a developing solvent. Method III: Cottonseed oil coated paper using absolute methanol–water (75:25) as a developing solvent.

$$R_{PG} = \frac{\text{Distance moved by antioxidant}}{\text{Distance moved by propyl gallate}} \times 100$$

Antioxidant	*Method I*	*Method II*	*Method III*
PG	100	100	100
NDGA	81	74	100
BHA	65	11	75
BHT	38	2	11
DPPD	34	2	20
Santoquin	65	6	58
DTBH	60	5	84
α-Tocopherol	19	0	0

It appears that as yet, no methods are available for the quantitative determination of PG and NDGA when they occur in a mixture[7]. The results presented herein would suggest that these two antioxidants can be determined in a mixture if paper chromatography were employed as a means of separation. Method II would probably be preferable for this purpose, since it gave the widest separation of PG and NDGA while the other antioxidants remained near the origin.

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SUMMARY

Three new methods were described for the paper chromatographic separation of various antioxidants. One utilized partially acetylated filter paper while the other two employed paper coated with cottonseed oil. A simple procedure for the preparation of partially acetylated filter paper was also described. Of particular interest was the complete separation of the four food-approved antioxidants (BHA, BHT, PG and NDGA) by unidimensional chromatography.

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CHROMATOGRAPHY OF PROTEINS ON CELITE ION-EXCHANGE RESINS

I. PREPARATION OF THE RESINS AND A STUDY OF THE CHROMATOGRAPHIC BEHAVIOUR OF CYTOCHROME *c*

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INTRODUCTION

Most of the adsorbents used for the chromatography of low molecular weight substances are not suitable for the chromatography of proteins. The main reason for this probably lies in the ease of denaturation of proteins at interfaces, although in some instances adsorbents have proved unsatisfactory for the chromatography of proteins because their capacity to adsorb proteins was too low. Due to their size, proteins are unable to penetrate into the finer pores of an adsorbent. Interaction between protein and resin takes place mainly on the surface of the adsorbent particles with a result that only adsorbents with large surface areas per gram are able to adsorb appreciable quantities of protein.

In the field of ion-exchange chromatography, the most successful adsorbent so far has been a finely divided form (XE-64) of the weakly acidic ion-exchange resin, Amberlite IRC-50. Several basic proteins[1] and the neutral proteins, haemoglobin[2] and myoglobin[3] were successfully subjected to chromatography on this resin. Ion-exchange resins prepared from cross-linked polystyrene have proved, in general, unsuitable for the chromatography of proteins because of their low capacity for protein[4]. In fact, the low capacity of polystyrene resins for protein was used to advantage for the separation of amino acids and peptides from proteins[5,6] and for the desalting of proteins[7]. However, there are reports in the literature of the use of resins derived from polystyrene for the chromatography of proteins. Columns of Dowex 50 were used in the purification of prostatic phosphatase[8] and for the separation of chymotrypsin and chymotrypsinogen[9], and a number of proteins were subjected to chromatography on columns of the anion-exchange resin, Dowex 2, by BOMAN AND WESTLUND[10]. But BOMAN AND WESTLUND[10] conclude from their experiments that the greatest shortcoming of Dowex 2 for the separation of proteins is its low capacity for protein.

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A number of ion-exchange adsorbents with high capacities for protein have been derived from cellulose[11]. Columns of these cellulose ion-exchange resins were used for the purification of some enzymes and in the fractionation of proteins from serum[12].

In the work reported here, ion-exchange resins with high capacities for protein were prepared by coating particles of a diatomaceous earth with ion-exchange materials. This communication describes preparations of a carboxylic acid ion-exchange resin and a sulphonic acid ion-exchange resin, but the method is also applicable to the preparation of anion-exchange resins. A preliminary account of the preparation of the sulphonic acid resin and its use in the chromatography of insulin were published previously[13]. A composite Celite–sulphonated polystyrene ion-exchange resin, with a low degree of cross-linking was prepared in this laboratory by FEITELSON AND PARTRIDGE[14]. This resin was specially designed for the chromatographic separation of large peptides.

The preparations of a weakly acidic ion-exchange resin and a strongly acidic ion-exchange resin, both with high capacity for protein offered a good opportunity for a detailed study of the behaviour of a particular protein on both types of resin under identical conditions of pH and cation concentration.

The protein chosen for study was cytochrome *c* as it was shown previously[2] that this protein could be successfully subjected to chromatography on columns of Amberlite IRC-50 over ranges of pH and cation concentration.

MATERIALS

Diatomaceous earth. This was Celite 545 obtained from John Manville and Co. Ltd., Artillery Row, London, S.W.1. Fines were removed by suspending the material in water, allowing it to settle for 30 min and removing the supernatant suspension. This process was repeated several times until the supernatant was clear after a settling time of 30 min. The product was dried in an oven at 105°. Celite, treated in this manner was used for the preparation of the carboxylic acid resin, but for the preparation of the sulphonic acid resin the Celite was made water-repellent by treatment with dichloro-dimethyl-silane[15].

Styrene. The commercial material contains a polymerisation inhibitor which was removed by shaking 4 or 5 times with 10% NaOH, followed by washing with water until the aqueous phase was no longer alkaline. The styrene was dried over anhydrous Na_2SO_4.

Divinyl-benzene. The commercial material which contains approximately 50% divinyl-benzene was used. The inhibitor was removed as described for styrene.

Methacrylic acid. This was purified by distillation under reduced pressure.

Cytochrome c. The material prepared from horse heart extracts by the method of KEILIN AND HARTREE[16] was purified by chromatography on columns of Amberlite IRC-50. The oxidised fraction was used in the chromatographic experiments.

METHODS

Preparation of Celite–carboxylic acid ion-exchange resin

The ion-exchange material was polymethacrylic acid, cross-linked with divinyl-benzene. Particles of Celite were coated with this material by heating a methanolic solution of methacrylic acid and divinyl-benzene in the presence of Celite and a suitable catalyst such as benzoyl peroxide. The method of polymerisation was similar to that used for the formation of vinyl polymers in wool[19,20] and the amount of polymethacrylic acid deposited on the Celite was controlled by the concentration of methacrylic acid in the methanol. The following procedure gave a product which contained 7.5% (w/w) polymethacrylic acid to Celite. The nominal degree of cross-linking was 10%. Benzoyl peroxide (0.1 g) was dissolved in a small quantity of benzene (1–2 ml) and added to methyl alcohol (75 ml). To this solution was added methacrylic acid (6.5 ml) and divinyl-benzene (1.2 ml) and the resulting mixture was stirred into dry Celite (50 g) to give a homogeneous mixture. Polymerisation was carried out in a sealed tube at 80° for 24 h. The product was washed thoroughly with methyl alcohol followed by water and dried at 105°. The total capacity of the resin for Na^+ was determined by reacting samples of resin (0.1 g) with 0.01 *N* NaOH (25 ml) and allowing to stand for several hours with occasional shaking. Aliquots (10.0 ml) of the supernatants were back-titrated with 0.01 *N* HCl using a mixture of methyl red and methylene blue as indicator. The capacity of the above preparation for Na^+ was 0.69 m.equiv./g dry resin, compared with 9.2 for Amberlite IRC-50.

The polymer was found to be uniformly spread over the particles of Celite. A small amount of the new resin was treated with the dye, basic fuchsin, and after thoroughly washing with water the particles were examined under the microscope. Dye was found to be evenly distributed over the particles: untreated Celite showed no uptake of dye. The resin was purified by alternate treatments with 0.1 *N* NaOH and 0.1 *N* HCl and it was finally converted to its sodium form with 0.1 *N* NaOH.

Preparation of Celite–sulphonic acid ion-exchange resin

The ion-exchange material was cross-linked sulphonated polystyrene. Celite was coated with cross-linked polystyrene by a method similar to that described for the carboxylic acid resin. However, in experiments with untreated Celite, polymer was formed preferentially in the methyl alcohol and it was difficult to obtain a stable film of polymer on the Celite particles. This difficulty was overcome by using Celite which had been made water-repellent by treatment with dichloro-dimethyl-silane. As in the preparation of the carboxylic acid resin, the amount of the polymer on the Celite was controlled, to a large extent, by the concentration of monomer in the methyl alcohol. Celite coated with 5% (w/w) cross-linked sulphonated polystyrene was used in the experiments reported in this paper.

Benzoyl peroxide (0.07 g) was dissolved in benzene (5 ml) and then methyl alcohol (10 ml) was added. This solution was added to methyl alcohol (60 ml), containing styrene (6.0 ml) and divinyl-benzene (0.6 ml) and the resulting mixture was stirred into 45 g of water-repellent Celite. The mixture was placed in a glass tube, the neck of which was partly drawn out. The tube was immersed in liquid oxygen and evacuated. Nitrogen was admitted and the tube was re-evacuated and nitrogen admitted again. The nitrogen filled tube was sealed off and placed in an oven at 65–70° for 7 days. The product of the polymerisation was washed thoroughly with methyl alcohol, followed by water, and after drying, it was sulphonated at 100° in concentrated sulphuric acid with silver sulphate as catalyst[21]. The sulphonated material was washed with repeated changes of distilled water until free from acid. The capacity of the resin for Na^+ was 0.28 m.equiv./g dry resin; the nominal content of divinyl-benzene was 5% of the weight of styrene. The sulphonic acid resin was purified by alternate treatments with 0.05 N NaOH and 0.1 N HCl and finally converted to its sodium form with 0.05 N NaOH.

Preparation and operation of columns

Purified resin was suspended in the buffer used for developing the chromatogram, allowed to settle for 1 h and the supernatant removed. This procedure was repeated 2 or 3 times until the supernatant was clear after a settling time of 1 h. The procedures employed in packing and operating the columns were the same as described previously[2], except that the columns were packed under a pressure of 5–10 cm Hg. The experiments with cytochrome *c* were carried out in jacketed columns at 25°.

0.3 ml samples containing 1–3 mg of purified cytochrome *c* were chromatographed on individual columns of the Celite–sulphonic acid resin, Celite–carboxylic acid resin and also on untreated Celite. The diameter of the columns was 0.9 cm and the heights of resin varied from 6 to 10 cm. In each experiment, the total volume of effluent, $V + Ve$[2], which passed through the column from the time of application of the sample until the maximum concentration of cytochrome appeared in the effluent was determined and then corrected to a standard resin height of 10 cm. The rate of flow of buffer through the column was 1 ml/h and the effluent was collected in 0.3 ml fractions.

Buffers

The effect of Na^+ concentration on the elution of cytochrome *c* from each of the coated Celite resins and from untreated Celite was studied at a constant pH of 7.0. Buffers contained 50.0 g of $Na_2HPO_4 \cdot 12H_2O$ and 8.6 g of $NaH_2PO_4 \cdot 2H_2O$/litre. NaCl was added to bring the Na^+ concentration to the required level and the pH was adjusted to pH 7.0 with N NaOH. The effect of pH on the elution of cytochrome *c* was studied at a constant Na^+ concentration of 1.0 g ions/litre. Between pH 5 and pH 8, citrate buffers were used, from pH 6 to pH 8 phosphate, and from pH 9 to pH 10 borate buffers. In all cases NaCl was added to bring the total concentration of Na^+ to 1.0 g ions/litre.

Analysis of effluent

Cytochrome *c* in the effluent was determined photometrically at 407 mμ in a Hilger "Uvispec" spectrophotometer.

RESULTS

Figs. 1 and 2 show the effects of pH and sodium ion concentration on the elution of cytochrome *c* from columns of Celite coated with cross-linked polymethacrylic acid (Cel-MX), Celite coated with cross-linked sulphonated polystyrene (Cel-SPX), and untreated Celite. In the experiments where the pH was varied, the Na^+ concentration was 1.0 g ions/litre and in the experiments where the Na^+ concentration was varied, the pH was 7.0.

The behaviour of cytochrome *c* on columns of Cel-MX was very similar to its behaviour on columns of Amberlite IRC-50[2]. In the pH range 5.5–6.5 the adsorption of cytochrome *c* was sharply dependent on the pH; a large increase in the adsorption occurred at pH values below 6 and under still more acidic conditions the cytochrome band remained stationary at the top of the column. In the pH range 7–9 the adsorption of protein was almost constant and it decreased slightly as the pH approached the isoelectric point of cytochrome *c*. As in the experiments with IRC-50 the adsorption of cytochrome *c* was dependent on the Na^+ concentration of the eluting buffer but the concentration of Na^+ required to elute cytochrome *c* from columns of Cel-MX at pH 7 was considerably higher than that required to elute the protein from columns of IRC-50. For example, with a column of IRC-50 and an eluting buffer of pH 7.0 and Na^+ concentration 0.34 g ions/litre the cytochrome band moved with an $R_F = 0.4$, but for the same rate of movement on columns of Cel-MX, a buffer of a Na^+ concentration 0.7 g ions/litre was required.

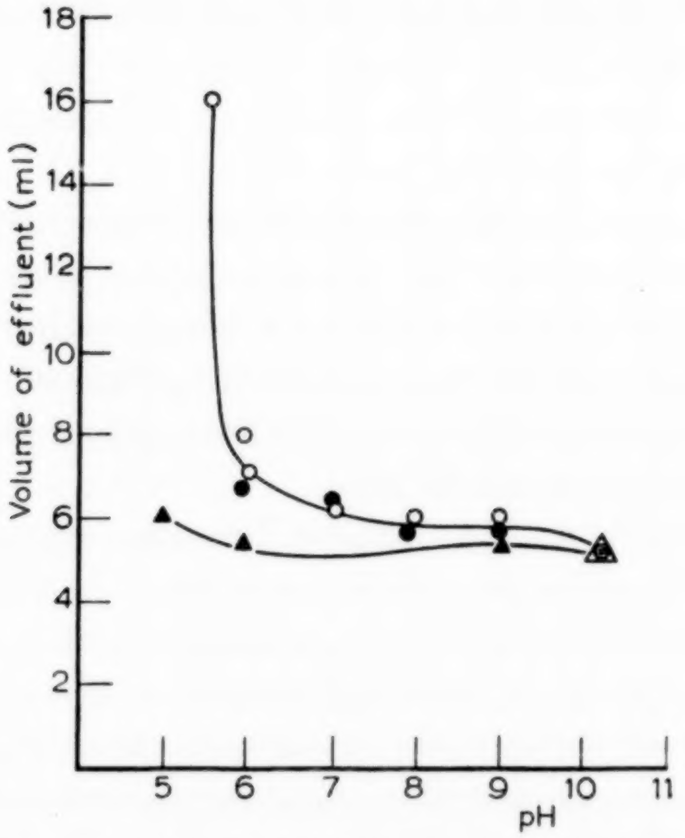

Fig. 1. Effect of pH on the elution of cytochrome *c* from columns of ion-exchange resin 10 × 0.9 cm. -○-○-○- Cel-SPX ion-exchange resin; -●-●-●- Cel-MX ion-exchange resin; -▲-▲-▲- Celite 545. Na^+ concentration 1.0 g ions/litre.

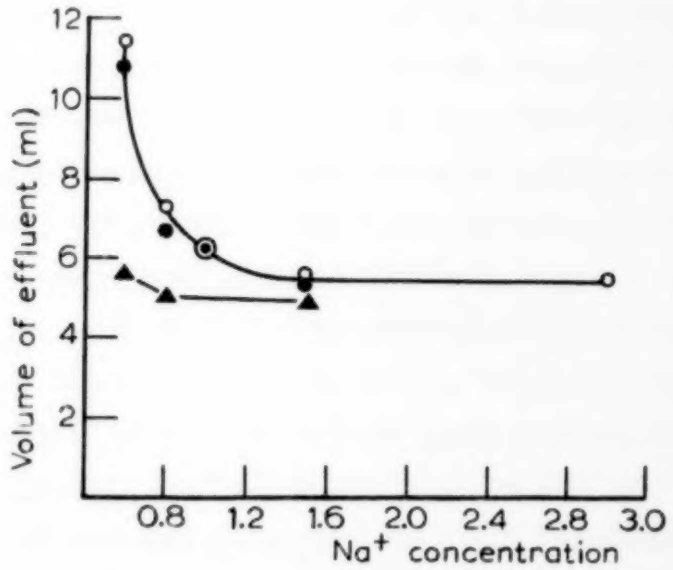

Fig. 2. Effect of sodium ion concentration on the elution of cytochrome *c* at pH 7.0 from columns of ion-exchange resins. -○-○-○- Cel-SPX ion-exchange resin; -●-●-●- Cel-MX ion-exchange resin; -▲-▲-▲- Celite 545.

The variations with pH and Na$^+$ concentration in the rate of movement of cytochrome *c* down columns of the sulphonic acid resin were almost identical with the variations observed on columns of Cel-MX. Similar changes in the adsorption of cytochrome *c* on both resins with Na$^+$ concentration at pH 7 were more or less expected since both resins are highly charged at this pH, but a surprising feature of the work was the rapid rise in the adsorption of cytochrome *c* on the sulphonic acid resin at pH values below 6.

Reversibility of the adsorption of cytochrome c

Although the R_F values of cytochrome *c* on columns of the carboxylic acid resin and the sulphonic acid resin were nearly identical, the shapes of the elution peaks on the two resins were not the same. Whereas the bands of cytochrome *c* on the carboxylic acid resin were almost symmetrical, the bands on the sulphonic acid resin showed tailing, particularly at R_F values < 0.5. This indicates that there was some irreversible binding of cytochrome *c* on the columns of the sulphonic acid resin. Thus, for the chromatography of a basic protein, such as cytochrome *c*, a carboxylic acid resin appears preferable to a sulphonic acid resin.

Untreated Celite showed a small adsorption for cytochrome *c* at pH 7 and Na$^+$ concentration 1.0 g ions/litre and this adsorption increased only slightly at low concentrations of Na$^+$ or at pH values below 6 (Figs. 1 and 2).

Gradient elution analysis

Weakly acidic ion-exchange resins, like IRC-50, with a high capacity for sodium ions, are not particularly suitable for chromatographic experiments involving either stepwise or gradual changes in the pH or sodium ion concentration of the eluting buffer, in the pH range 5–7. These resins are very strong buffers in this pH range and

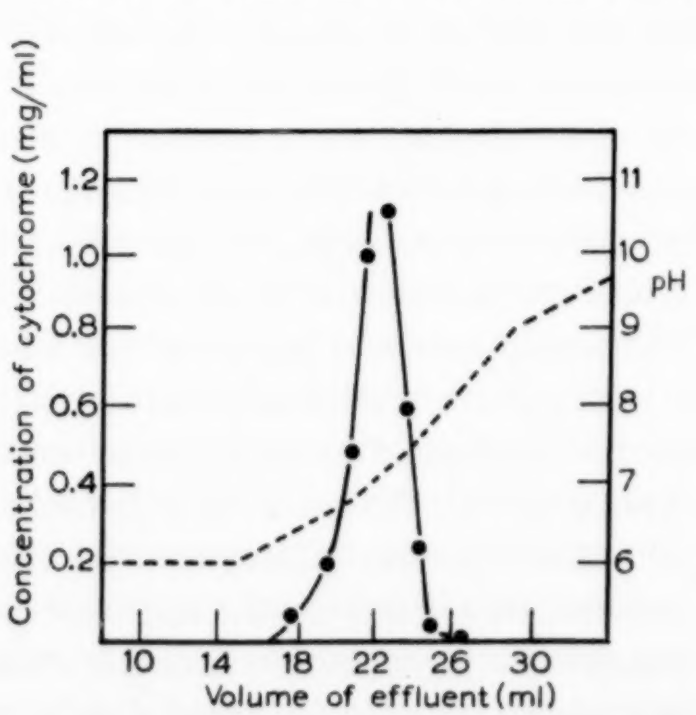

Fig. 3. Chromatogram of cytochrome *c* on a column of Cel-MX ion-exchange resin, 4.2 × 1.8 cm. An eluting buffer of gradually increasing pH was used to elute the cytochrome *c*. Amount on column: 3.8 mg. Dotted line shows the calculated pH values of effluent.

large volumes of eluting buffer are required to effect changes in the pH of the effluent from columns of such resins. Because of its low buffering capacity per ml of column volume, compared with IRC-50, Cel-MX ion-exchange resin may be used to advantage where it is desired to elute the proteins by a stepwise or gradual increase in the pH or cation concentration of the eluting buffer.

As an example, Fig. 3 shows the chromatogram obtained when purified cytochrome *c* was eluted from a column of Cel-MX by a buffer of gradually increasing pH. It may be noted that the rear of the cytochrome *c* band is sharper than the front, due to the higher pH gradient in the vicinity of the rear boundary. Although the pH of the eluting buffer applied to the column varied linearly with the volume passed through the column over the pH range 6–11, the pH of the emerging buffer increased more steeply with volume as the pH rose above 7, due to the progressively lower buffering capacity of the resin as the resin becomes saturated with sodium ions. It is possible to calculate the pH of the buffer emerging from the column from a knowledge of the titration curves of both resin and buffer and the pH of the buffer applied to the column[4]. The calculated values agree well with those determined experimentally.

Capacity of the Celite ion-exchange resins for protein

In the experiments with cytochrome *c* and insulin, loads of 5–10 mg protein/cm^2 cross-section of column were successfully chromatographed on the Celite ion-exchange resins.

DISCUSSION

Chromatography of cytochrome c

Previously, it was suggested that the large increase in the adsorption of cytochrome *c* on Amberlite IRC-50 at pH values below 6 was due to uncharged carboxyl groups on the resin, since it was observed that the rapid rise in the elution volumes of cytochrome *c* occurred over the same range of pH as the fall in amount of sodium ions retained by the resin from buffers of comparable concentration[2]. A similar hypothesis would explain the observed behaviour of cytochrome *c* on columns of Cel-MX.

However, the experiments with the sulphonic acid resin show that cytochrome *c* is adsorbed strongly at pH values below 6 in the absence of uncharged carboxyl groups on the resin. With the sulphonic acid resin, the high adsorption of cytochrome *c* is not due to a change with pH in the ionization of the groups on the resin. It now seems unlikely, therefore, that uncharged carboxyl groups on the resin are responsible for the increased adsorption of cytochrome *c* on columns of IRC-50 or Cel-MX, although they may contribute to the overall adsorption. The factors mainly responsible for the adsorption of cytochrome *c* either on the sulphonic acid or carboxylic acid resin are most likely changes in the configuration or ionization of the protein molecule.

The rapid rise in the adsorption of cytochrome *c* on both carboxylic acid and sulphonic acid resins at pH values below 6 is probably due to an increase in the number of points of attachment between protein and resin at these pH values. An increase in

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the binding of protein to resin could result from a simple increase in the nett charge on the protein molecule or it could arise from configurational changes in the protein molecule, since rearrangement of the protein molecule may lead to the formation of new points of attachment between protein and resin. It follows from a consideration of mass action that there will be a large increase in the adsorption of cytochrome *c* on lowering the pH from 7 to 5, if the number of points of attachment between protein and resin is increased while the sodium ion concentration in the ambient phase remains constant. For example, an increase in the number of binding points from (say) 5 to 8 could result in a very large increase in elution volume[2].

Chromatography of acidic proteins

Although cytochrome *c* showed similar adsorptions on both the carboxylic acid and sulphonic acid resins, the acidic protein insulin behaved in a different way[22]. Whereas insulin was reversibly adsorbed on Cel-SPX at pH 3.4 from an acetate buffer of sodium ion concentration 1.0 g ions/litre, with a distribution coefficient of approximately 1, it was irreversibly adsorbed on the carboxylic acid resin, Cel-MX.This would suggest that in the case of insulin uncharged carboxyl groups are playing a prominent role in the adsorption of the protein.

Previous studies on the adsorption of proteins by Amberlite IRC-50[4] indicated that this carboxylic acid resin was not suitable generally for the chromatography of acidic proteins. The proteins studied either were not adsorbed by the resin or they were strongly adsorbed, depending on the pH of the eluting buffer. All the proteins were strongly adsorbed at pH values below 5, where the ionization of the carboxyl groups on the resin was suppressed. It seemed to us that a sulphonic acid resin with a high capacity for protein may prove a suitable adsorbent for the chromatography of acidic proteins, as the sulphonic acid groups are fully ionized within the pH range 1–12.

Experiments with insulin showed that the Celite-SPX ion-exchange resin was a suitable adsorbent for the chromatography of a relatively stable, acidic protein of low molecular weight. However, studies with a number of other proteins such as bovine serum albumin, bovine haemoglobin, lactoglobulin and tobacco mosaic virus showed that the chromatographic behaviour of these proteins on columns of Cel-SPX was sharply dependent on the pH and cation concentration of the eluting buffer in the pH range 5–6. The experiments with tobacco mosaic virus will be reported in a future publication. Thus, the behaviour of these acidic proteins on the sulphonic acid resin closely resembled their behaviour on a carboxylic acid resin such as Amberlite IRC-50. Conditions for the successful chromatography of an acidic protein on Cel-SPX may therefore be fairly sharply defined and the ideal pH and salt concentration should be evaluated for each protein by carrying out test tube experiments[23].

The fact that a protein can be eluted from the resin only over a narrow range of pH and Na^+ concentration and that this range varies from protein to protein makes the method of elution with a buffer of constant composition unsuitable for the separation of all proteins in a mixture. A buffer which elutes one of the proteins in the mixture is unlikely to effect a satisfactory separation of all the proteins in the mixture. The elution of all the proteins, individually, will require a series of buffers of increasing eluting power or, alternatively, a buffer of gradually increasing eluting power[24]. Both the stepwise elution and gradient elution techniques were used by Tiselius, Hjerten and Levin[25] and by Boman and Westlund[10] in their experiments on the separation of proteins on calcium phosphate and ion-exchange resins, respectively. Although elution of proteins by stepwise changes in the buffer or by the gradient method offers many practical advantages over elution with a buffer of constant composition, great care must be exercised in interpreting the results where the former methods are used. This point is discussed in greater detail in the following paper[22].

As postulated for the basic protein cytochrome *c*, the rapid change in the adsorption of the acidic proteins on Cel-SPX with pH of buffer is probably due to an increase in the number of points of attachment between protein and resin. It is postulated that the increased binding of cytochrome *c* to the resins could result either from an increase in the nett charge on the protein or from configurational changes in the protein. With the acidic proteins, the increased binding is probably due to configurational changes in the protein molecules, since the adsorption of the acidic proteins changes so markedly over narrow ranges of pH. It appears that the adsorption of a protein on an ion-exchange resin can be a very sensitive method for distinguishing between protein molecules. Changes in the configuration of a protein may result in dramatic changes in the adsorption of the protein. If a particular configurational change in a protein molecule is sharply dependent on pH, then the adsorption of that protein may be sharply dependent on pH. This situation bears comparison with the acid denaturation of haemoglobin. From studies on the kinetics of denaturation of haemoglobin, Steinhardt and Zaiser[26] were led to the view that the combination of hydrogen ions with a small number of "trigger groups" in the protein sufficed to initiate its denaturation as measured by loss of solubility at the isoelectric point and by the all or nothing appearance in each molecule of 36 acid binding groups.

The adsorption and desorption of a protein on a cation-exchange resin may possibly be described by the following scheme.

Adsorption $P_1 + xH^+ \rightleftharpoons P_1H_x \rightarrow P_2H_x \rightarrow \text{Resin } P_2H_x$

Desorption $\text{Resin } P_2H_x - xH^+ \rightarrow \text{Resin } P_2 \rightarrow \text{Resin } P_1 \rightarrow P_1$

The protein molecule represented by state P_1 is not adsorbed by the resin, but combination with a few protons initiates a change in the configuration of the protein molecule (P_2H_x) and then adsorption of the protein by the ion-exchange resin takes place. The desorption of the protein is brought about by removing protons from the adsorbed protein. This removal of protons enables the protein to assume the configuration which is not adsorbed by the resin. This hypothesis, no doubt, represents an over-simplified picture, as it is possible to visualize a number of ionization states for the protein molecule, resulting in configurational changes in the protein. However, such changes in the configuration of a protein may explain the dramatic changes in the adsorption behaviour of proteins with small changes in pH.

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SUMMARY

1. Ion-exchange resins with a high capacity for protein were prepared by coating particles of the diatomaceous earth, Celite 545, with ion-exchange materials. Preparations of a carboxylic acid resin and a sulphonic acid resin are described.

2. A detailed study of the behaviour of cytochrome *c* on both resins was made under varying conditions of pH and cation concentration. The surprising feature of the results was the very high adsorption of cytochrome *c* on the sulphonic acid resin at pH values below 6.

3. A possible hypothesis to explain the high adsorption of proteins on cation-exchange resins in the region of pH 5 is outlined.

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CHROMATOGRAPHY OF PROTEINS ON CELITE ION-EXCHANGE RESINS

II. CHROMATOGRAPHY OF INSULIN ON A CELITE-SULPHONIC ACID ION-EXCHANGE RESIN

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INTRODUCTION

The preparation of a sulphonic acid ion-exchange resin with a high capacity for protein is described in the preceding paper[1]. Particles of the diatomaceous earth, Celite 545, were coated with a thin film of cross-linked sulphonated polystyrene to produce a resin with a large surface area.

This paper describes the application of columns of the Celite–cross-linked sulphonated polystyrene (Cel-SPX) to a study of the homogeneity of crystallized insulin. A preliminary account of the work was published previously[2].

MATERIALS AND METHODS

Ion-exchange resin

The Cel-SPX ion-exchange resin was prepared as described in the preceding paper[1].

Dowex 50

For comparison with its behaviour on columns of Cel-SPX, 6 times crystallized insulin also was chromatographed on a column consisting of a mixture of Celite 545 and the sulphonic acid ion-exchange resin, Dowex 50, in a finely divided state. Beads of Dowex 50 (X8) were ground in a laboratory hammer mill. Material which passed through a 200 mesh/in. sieve was treated several times with hot 2 *N* NaOH, washed with water and sieved again in the wet condition. Material which passed a 200 mesh/in. sieve was dried and mixed with Celite 545 in the ratio 1:20.

Insulin

Most of the insulin samples, either crude or 6 times crystallized, were generously provided by Boots Pure Drug Co. A sample of crystallized insulin which had been prepared by Dr. LENS[3] was kindly supplied by Dr. F. SANGER.

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Preparation and operation of the columns

The procedures employed in packing and operating the columns were the same as described previously[4]. Most of the experiments were carried out with columns of Cel-SPX, 0.9 cm in diameter, and 12 cm high. 0.4 ml samples containing 3–4 mg of insulin were added to the columns and the effluent collected in 1.0 ml fractions. The rate of flow of buffer through the column was 1.0 ml/h. The choice of buffers for the chromatography of insulin was limited by the low solubility of insulin. Insulin is insoluble in the pH range 4–7 and even in the pH range 2–4 it is not soluble in all buffers. An acetate buffer of the following composition was used to elute insulin from the columns: 65.3 g $CH_3COONa \cdot 3H_2O$, 30.5 g NaCl and 300 ml glacial acetic acid/l. The pH of the buffer was 3.39 and the Na^+ concentration 1.0 g ions/l.

Protein concentration in the effluent was measured photometrically at 276 mμ in a Hilger "Uvispec" spectrophotometer. A correction for extraneous absorption was made from simultaneous measurements of the optical density at 320 and 360 mμ, according to the method of BEAVEN AND HOLIDAY[5]. The line through the points at 320 and 360 mμ was extrapolated to 276 mμ and this formed a base line from which the optical density was measured.

Concentration of insulin in effluent

Before attempting to concentrate the insulin in the effluent, it was necessary to dialyse away most of the buffer salts. Normal dialysis against water, using a Visking cellophane sac, but without agitating the liquid inside or outside the sac proved unsatisfactory because of heavy losses of insulin from inside the sac during the time taken to remove 95% of the buffer salts. However, it was observed that most of the salt could be removed, without loss of insulin, by increasing the rate of dialysis. This was done by increasing the effective area of the sac in relation to the volume of liquid it contained, and by agitating the solution inside the sac.

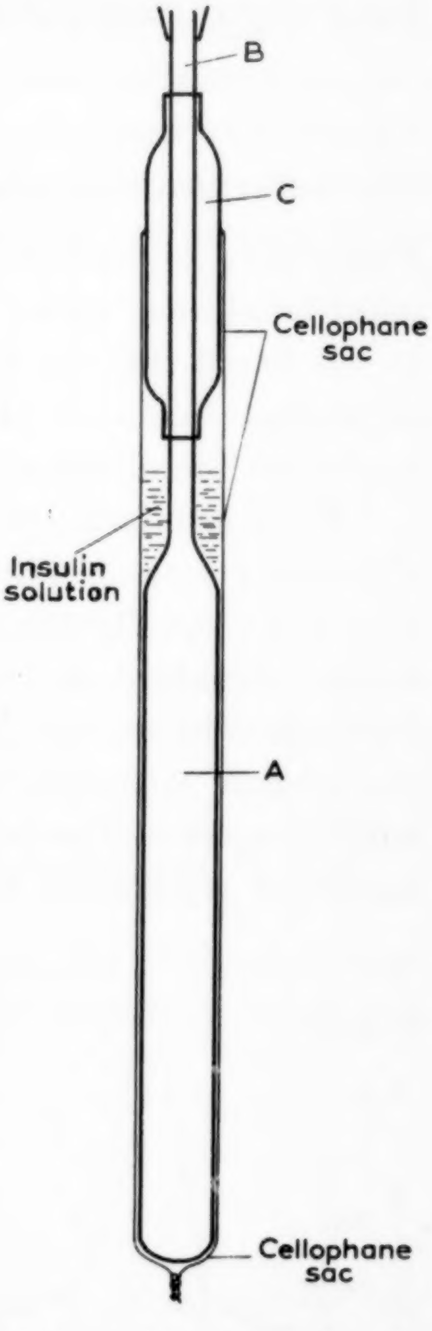

Fig. 1. Apparatus for the rapid dialysis of insulin solutions (see text).

The protein solution was agitated by a rotating glass tube, A (Fig. 1), which was sealed at one end and joined at the other to a length of glass tubing of narrower bore (B). B was connected to the shaft of a stirrer motor. A small length of glass tubing (C), drawn down at both ends, acted as a guide for the rotating tube as well as a support to which the cellophane sac was tied.

10 ml of effluent was placed in the sac, tube A was inserted and then connected

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to the motor. The sac was tied to C which was then lightly clamped. The sac was lowered into 5 l of distilled water and stirring commenced. The water outside the sac also was agitated by a magnetic stirrer. Dialysis was allowed to continue for 30 min with one change of water and during this period 95% of the salt was removed with negligible loss of insulin. The solution of insulin was concentrated by allowing it to stand over calcium chloride in a desiccator.

RESULTS

Chromatography of crystallized insulin

Distribution experiments were carried out in graduated centrifuge tubes[6] in order to select conditions under which the adsorption of insulin to Cel-SPX was reversible. It was found that the crystallized insulin was reversibly adsorbed on Cel-SPX from an acetate buffer of pH 3.39 and Na^+ concentration 1.0 g ions/l and under these conditions the distribution coefficient was favourable for chromatography.

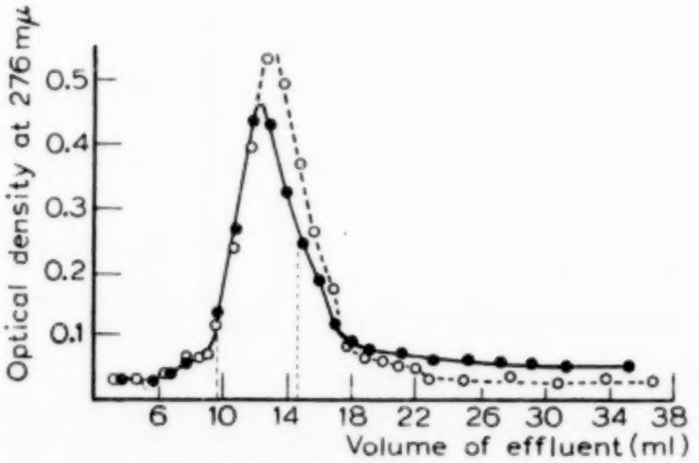

Fig. 2. Chromatogram of crystallized insulin on a column of Cel-SPX, 12.3 × 0.9 cm. Buffer: sodium acetate, pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. —●—●—●— 6 × crystallized insulin obtained from Boots Pure Drug Co. Amount on column 3.3 mg. --o--o--o-- Insulin crystallized by LENS. Amount on column 3.5 mg (for the main peak $R = 0.62$, $R_F = 0.34$).

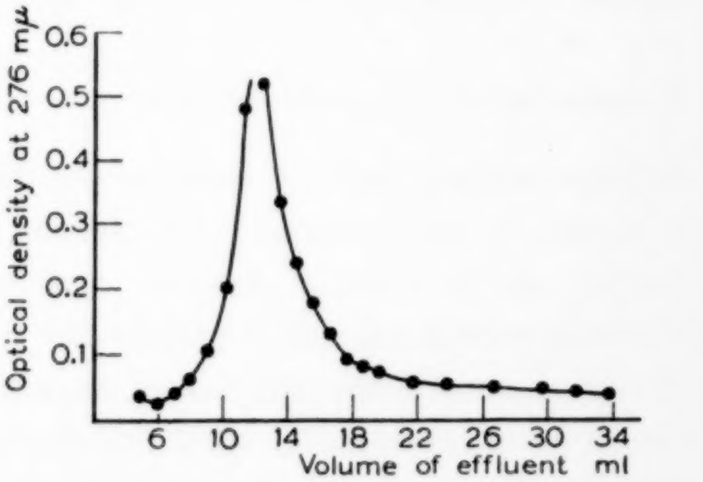

Fig. 3. Chromatogram of a crystallized insulin on a column of Cel-SPX, 12.3 × 0.9 cm. Buffer: sodium acetate pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. Amount on column 3.6 mg.

The continuous line in Fig. 2 shows the chromatogram obtained from a sample of 6 times crystallized insulin (Boots Insulin, Batch 9011G), using a column of Cel-SPX, 12.3 × 0.9 cm. The shape of the elution curve indicates that the sample of crystallized insulin contained at least two components. Furthermore, the recovery of protein from the column was 73%, showing that there was some adsorption of material on the column. Attempts were made to resolve the components shown in Fig. 2, but without success. The dotted line in Fig. 2 shows the chromatogram obtained with a sample of crystallized insulin which had been prepared by Dr. LENS[3]. In this case, the elution peak was almost symmetrical, showing that the material eluted from the column was essentially homogeneous; the recovery of protein from the column was 78%. Other samples of crystallized insulin, kindly supplied by Dr. DICKINSON, were chromatographed. Most samples showed the presence of a second component, but one sample which had been purified on columns of calcium phosphate[7] gave an almost symmetrical peak (Fig. 3).

Rechromatography of insulin

In order to obtain sufficient material for rechromatography, 35 mg of 6 times crystallized insulin (Boots) were chromatographed on a column 7.6 × 1.8 cm. A cut which corresponded to the dotted vertical lines in Fig. 2 was made and the insulin concentrated by rapid dialysis, followed by freezing and allowing to stand over calcium chloride. Rechromatography on a fresh column gave an elution curve which still showed the presence of two components (Fig. 4), but the recovery of protein (85%) was higher than in the original chromatogram. This inhomogeneity of the insulin on rechromatography was not surprising in view of the small degree of separation obtained in the first elution and the width of the cut.

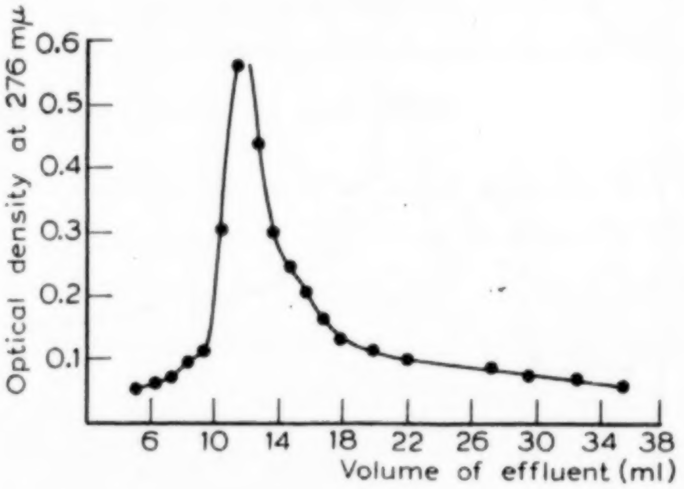

Fig. 4. Rechromatography of 6 × crystallized insulin on Cel-SPX. Column 12.3 × 0.9 cm. Buffer: sodium acetate, pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. Amount on column 3.3 mg.

Assay of insulin

Biological assay of insulin which had been eluted from a column of Cel-SPX was kindly carried out by Boots Pure Drug Co. The activity of the eluted insulin was 23.1 I.U./mg ($P = 0.95$, 20.16–26.68), a value not significantly different either from the activity of the crystallized insulin before chromatography (22.2 I.U./mg) or from the International Standard.

Chromatography of a preparation of crude insulin

A preparation of crude insulin was supplied by Dr. DICKINSON. This preparation was obtained at an intermediate stage of the manufacturing process and it represented the protein contained in the liquor for isoelectric precipitation. Fig. 5a shows the result obtained when the crude insulin was subjected to chromatography on a column of Cel-SPX, 12.3 × 0.9 cm under the same conditions as was used for the chromatography of crystallized insulin. A protein band emerged from the column before the insulin, but the separation of this protein from the insulin was incomplete. An improved separation was obtained on a longer column of Cel-SPX, 20.0 × 0.9 cm (Fig. 5b).

Attempts also were made to purify the crude preparation of insulin by chromatography on columns of Cel-SPX with stepwise changes in the eluting buffer. Stepwise elution of proteins from a column offers particular advantages for preparative work, provided conditions can be found for the complete separation of the protein being purified from the other proteins in the mixture, while retaining the particular protein or enzyme in a fully active state. Stepwise elution was particularly successful in the preparation of cytochrome c^8.

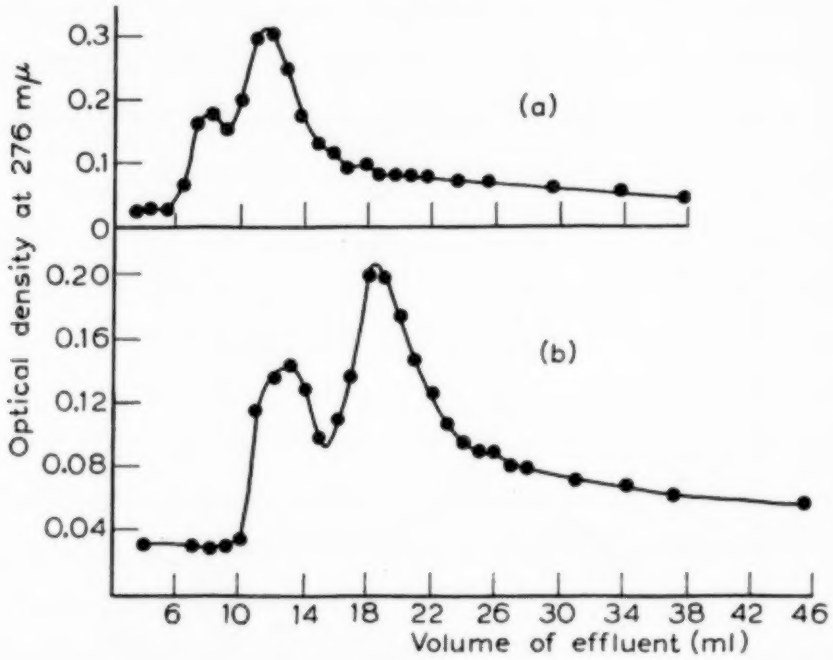

Fig. 5. Chromatograms of a preparation of crude insulin on Cel-SPX. (a) Column 12.3 × 0.9 cm. (b) 20.0 × 0.9 cm. Buffer: sodium acetate pH 3.39, Na^+ concn. 1.0 g ions/l. Temp. 2°. Amounts on column: approx. 3.5 mg.

In the experiments with the crude insulin the aim was to apply the protein mixture to the column in a buffer such that the insulin remained as a stationary band at the top of the column, while the contaminating proteins passed through the column. After washing the latter proteins from the column, it was hoped to elute the insulin in a fully active state by changing to a buffer of higher eluting power. Unfortunately, we were unable to find a buffer which would quantitatively elute the contaminating proteins from the column and, at the same time, leave the insulin completely adsorbed on the resin.

In one experiment, the preparation of crude insulin was applied to the column of Cel-SPX in a buffer of Na^+ concentration 0.60 g ions/l and pH 3.39, and the same buffer was passed through a column until 40 ml of effluent was collected. The buffer was then changed to one of Na^+ concentration 1.5 g ions/l and pH 3.39. At first sight, the result appeared satisfactory; two distinct peaks were obtained with a good base line between them. However, the amount of protein eluted by the first buffer was low, suggesting that a proportion of the contaminating proteins was adsorbed to the resin from the first buffer and eluted with the insulin when the buffer was changed. The protein peak eluted by the first buffer showed a small amount of tailing, which might suggest that some of the contaminating protein was spread out over the column.

When the starting buffer was increased in Na^+ concentration to 0.80 g ions/l, it was found that part of the insulin was eluted in a second broad band by the starting buffer. The remainder of the insulin was eluted by the second buffer of Na^+ concentration 1.5 g ions/l. The results of these two experiments show that great care must be exercised in interpreting the results of protein chromatography if stepwise elution is used.

Chromatography of insulin on the carboxylic acid ion-exchange resin

Insulin was irreversibly adsorbed on a column of Celite, coated with cross-linked methacrylic acid (Cel-MX) from an acetate buffer of pH 3.4 and Na^+ concentration 1.0 g ions/l.

Chromatography of insulin on Dowex 50 and Celite

Finely ground Dowex 50 was mixed with Celite 545 in the ratio 1:20 (w/w). The exchange capacity/g of the mixture for Na^+ was similar to the exchange capacity/g of Cel-SPX. The open circles in Fig. 6 show the result obtained when a sample of 6 times crystallized insulin was passed through a column of Dowex 50, mixed with Celite, with an acetate buffer of pH 3.4 and Na^+ concentration 1.0 g ions/l. The closed circles show the result obtained when insulin was passed through a column of Celite 545. There was no significant retardation of the insulin band in either experiment, and the yield of insulin from the columns was quantitative. Comparison of the two curves in Fig. 6 shows that Dowex 50 even in a very finely divided form has a negligible capacity to adsorb insulin. The success of Cel-SPX as a resin for the chromatography of insulin is probably due to the large surface area of ion-exchange material presented by the coated diatom skeletons. On the other hand, particles of Dowex 50 presumably have a relatively small surface area as they exhibit smooth glass-like surfaces when viewed under the microscope.

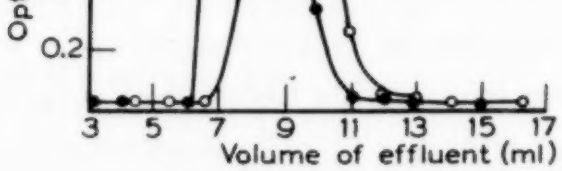

Fig. 6. -o-o-o- Chromatogram of 6 × crystallized insulin on a column of Dowex 50, mixed with Celite 545. Column 13.8 × 0.9 cm. -•-•-•- Chromatogram of 6 × crystallized insulin on a column of Celite 545, 12.0 × 0.9 cm. Buffer: sodium acetate pH 3.39, Na^+ conc. 1.0 g ions/l. Temp. 2°. Amount on columns 3.5 mg.

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DISCUSSION

There is evidence from the chromatograms for more than one component in all except one of the samples of insulin, which were investigated. With the samples of crystallized insulin, the separation of the components was insufficient to permit isolation of any component in a pure state. Therefore, no attempt was made to distinguish between the components, either by physical or chemical examination.

Our results are in general agreement with those of HARFENIST AND CRAIG[9], who showed that a number of samples of crystallized insulin from various sources could be split into two components by counter current distribution between *n*-butanol and dichloroacetic acid, although the proportion of the two components varied considerably among the samples investigated. Beef insulin from the Eli Lilly Co. contained a larger proportion of the minor component than did beef insulin obtained from Boots Pure Drug Co. HARFENIST[10] examined the amino acid composition of the two components of beef insulin from Eli Lilly Co. and it appeared that the only difference between the components was in their amide content. One component had 6 amide groups per molecule and the other 5. As HARFENIST points out, it is not improbable that one of the components is an artifact of the method of preparation.

Conversely, PORTER[11] in his studies on the partition chromatography of insulin on kieselguhr columns found that the insulin peak behaved as a single component. DICKINSON[7] used chromatography on calcium phosphate columns to distinguish between freshly prepared solutions of crystallized insulin and solutions which had been stored under various conditions. Freshly prepared solutions gave a single peak on the chromatograms with only slight tailing, while stored solutions showed the presence of a second component, which increased in amount with time of storage. DICKINSON suggested that the appearance of a second component may have been due to molecular weight changes in the insulin.

Recovery of insulin

The recovery of the crystallized insulins from the columns varied from 70 to 80%, and it was increased by a second passage of insulin through the column. This indicates that there were small amounts of material present in the crystallised insulin which were strongly adsorbed to the resin during the first passage through the column. However, the recoveries of insulin on rechromatography were still not quantitative, showing that there was some irreversible binding of insulin to the resin even on the second passage through the column.

Gradient elution analysis

For reasons which are outlined in the preceding paper[1], stepwise or gradient elution procedures may have practical advantages in protein chromatography over the method of elution with a buffer of constant composition. However, the experiments with insulin, reported in this paper, emphasize the difficulties of interpretation in experiments where the eluting buffer is not constant. Proteins which show marked tailing on the column may appear in several peaks, as each change of the eluting buffer may desorb protein from the column. The appearance of a protein in several peaks may occur even when a buffer of gradually increasing eluting power is applied to the column. It is also difficult to draw conclusions about the homogeneity of a protein preparation, when stepwise elution is used, as several proteins may be eluted together with a change in the eluting medium.

The stepwise and gradient elution methods suffer from a further disadvantage, particularly if labile proteins are being subjected to chromatography. In either method it is usual to select a starting buffer of very low eluting power, so that all the proteins in the mixture being chromatographed are strongly adsorbed on the column. Very strong adsorption may bring about irreversible changes, including hydrolytic ones in a protein, whereas in the method of elution at constant composition the buffer is usually chosen so that the particular protein being investigated moves down the column with an $R_F > 0.5$ (BOARDMAN AND PARTRIDGE[4]).

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The author wishes to thank Dr. S. M. PARTRIDGE for his interest in this work and for helpful suggestions and Dr. F. SANGER F.R.S., for the gift of some of the insulin samples. The author is greatly indebted to Dr. W. DICKINSON of Boots Pure Drug Co. for the generous supply of insulin samples and for arranging the biological assays. This work was carried out during the tenure of an Imperial Chemical Industries Fellowship and I thank Dr. E. C. BATE-SMITH for the provision of laboratory facilities.

SUMMARY

1. Several samples of crystallized insulin and a preparation of crude insulin were subjected to chromatography on columns of a sulphonic acid ion-exchange resin.

2. The chromatograms showed the presence of more than one component in all except one of the samples of insulin which were investigated.

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GAS–LIQUID PARTITION CHROMATOGRAPHY OF ISOMERIC ALKYLCYCLOPENTENES AND ALKYLIDENECYCLOPENTANES

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The analysis of isomeric alkylcyclopentenes and alkylidenecyclopentanes is of interest in connection with the study of such reactions as the double bond migration[1,2] and the skeleton rearrangement[3] of cycloolefins. Further, cyclopentenes have been found in catalytically cracked gasolines[4,5].

Examination of the properties of the C_6 and C_7 cyclopentenes, listed in Table I, shows that the difference between the boiling points of two isomers (II and III) is 1.5° and that for others (I and IV; VI and VII) this difference is only 0.1°. Analysis by distillation of compounds whose boiling points are so close together is not practicable. By combining distillation with oxidation to the corresponding dibasic acids, the various isomers can be determined, but this procedure is laborious and not very accurate.

On the other hand, Raman[1] and infrared[4,6,7] spectra, and gas–liquid partition chromatography (G.L.P.C.) are sufficiently selective to permit qualitative and quantitative analysis of the different isomers. The present paper deals with the G.L.P.C. of mixtures containing:

(a) the isomeric methylcyclopentenes and methylenecyclopentane;

(b) the isomeric ethylcyclopentenes and ethylidenecyclopentane.

TURNER AND GARNER[2] have analysed mixtures of cyclopentenes by G. L. P. C. They have reported, however, that they could not separate such pairs as 1-methylcyclopentene (I) and methylenecyclopentane (IV), under the chromatographic conditions they employed and that they had to use infrared absorption instead.

Obviously a very selective phase was necessary for the separation of the mixtures of compounds studied. Silver nitrate–glycol, as the stationary phase, seemed to be suitable, particularly in view of its successful use in the analysis of the methylcyclohexene isomers[8].

Details of the procedure are described in the experimental section.

The results are given in Table I and Figs. 1 and 2. The relative retention volumes, which increase with the concentration of silver nitrate in the liquid phase, were determined for a practically saturated solution, containing 9.66 g $AgNO_3$/10 ml glycol. They were corrected for dead volume and expressed relative to toluene. It can be seen that the isomers of both the C_6 and the C_7 groups can be easily separated from

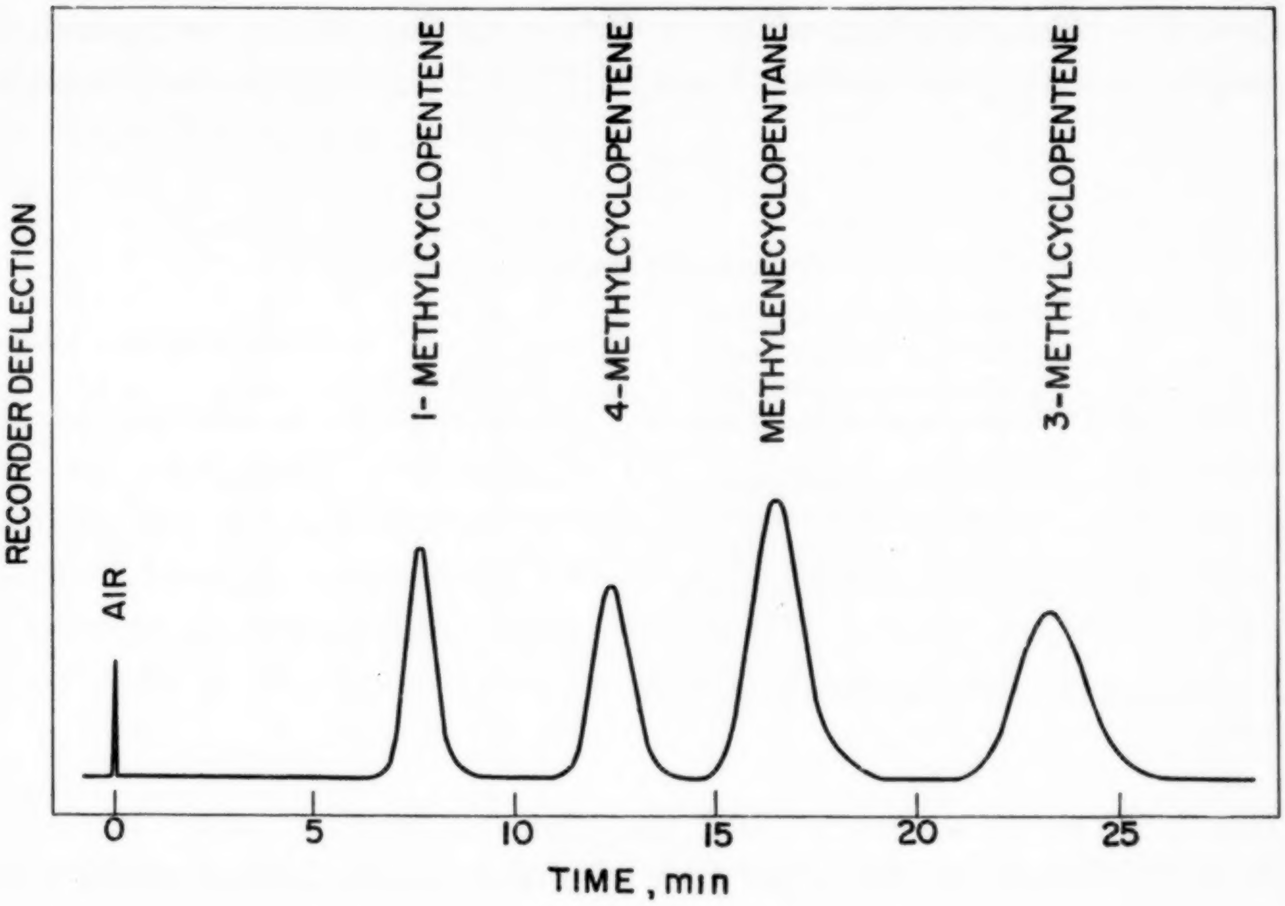

Fig. 1. Chromatography of a mixture of the three isomeric methylcyclopentenes and methylenecyclopentane on a column of 1 m with a saturated $AgNO_3$–glycol solution as the stationary phase at 30°; helium flow rate 36 ml/min.

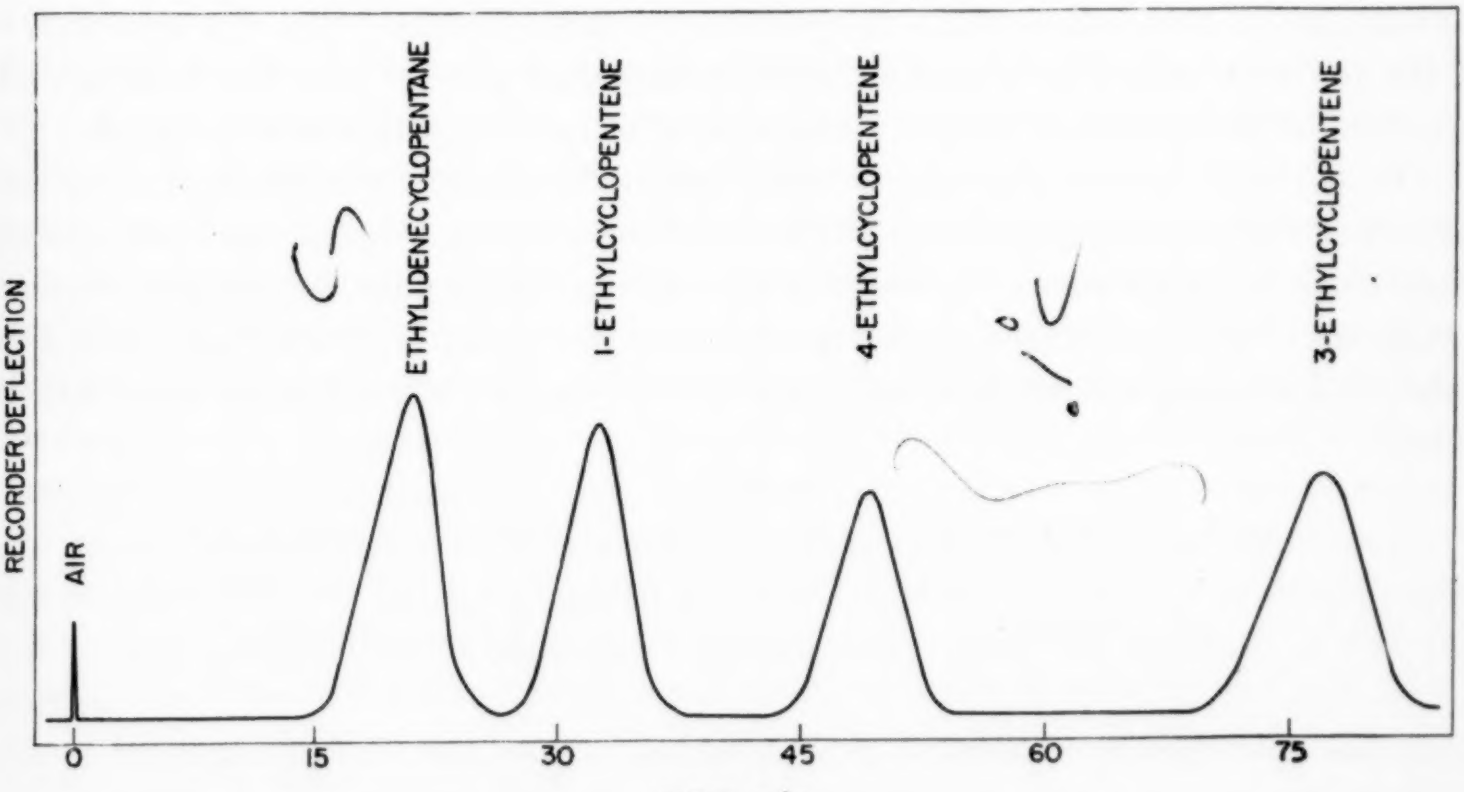

Fig. 2. Chromatography of a mixture of the three isomeric ethylcyclopentenes and ethylidenecyclopentane on a column of 2 m with a saturated $AgNO_3$–glycol solution as the stationary phase at 30°; helium flow rate 42 ml/min.

each other. The large difference of the retention volumes of the compounds whose boiling points are very close together (I and IV; VI and VII) is particularly remarkable.

TABLE I

No.	*Compound*	*B.p. (°C)/760 mm*	R_V/R_V *toluene**
I	1-Methylcyclopentene	75.8	0.75
II	3-Methylcyclopentene	65.0	2.35
III	4-Methylcyclopentene	66.5**	1.23
IV	Methylenecyclopentane	75.7	1.66
V	1-Ethylcyclopentene	106.3	1.30
VI	3-Ethylcyclopentene	98.1	3.37
VII	4-Ethylcyclopentene	98.2	2.12
VIII	Ethylidenecyclopentane	112.6	0.68

* Concentration of $AgNO_3$/glycol 9.66 g/10 ml.

** 735 mm.

It has been shown previously[8,9] that the complex-forming silver nitrate–glycol phase is more efficient in the separation of olefins whose boiling points are close together, than polar phases. The selectivity must be ascribed essentially to the variation of the stability of the silver ion–olefin complexes with structure. Inspection of Table I shows that in agreement with the findings of WINSTEIN AND LUCAS[10], the retention volumes depend on steric factors and decrease with the increase of the number of subsituents around the double bond (R_V of I $<$ II, III, and IV; R_V of V and VIII $<$ VI and VII).

On the other hand comparison of II with III, and of VI with VII shows that, for the same number of substituents, the retention volume increases with the asymmetry of the molecule and that therefore polar factors also play a role. The influence of structure on the retention volume is the subject of further work now in progress.

It has been shown[8] that the thermal conductivities of the methylcyclohexenes are sufficiently similar to permit quantitative determination without prior calibration, when using a katharometer as the detector and helium as the carrier gas. Similar results were obtained for the cyclopentenes as can be seen from the example given in Table II. The compositions were calculated by the method of integration as described before[8].

TABLE II

DEVIATION IN ANALYSIS OF A SYNTHETIC BLEND OF C_7 CYCLOPENTENES

Compound	*% by weight*		*Deviation*
	Weighed	*Found*	
1-Ethylcyclopentene	41.85	40.90	0.95
		42.15	0.30
3-Ethylcyclopentene	29.05	27.75	1.30
		27.60	1.45
Ethylidenecyclopentane	29.10	31.35	2.25
		30.25	1.15

References p. 410.

By using the method on a preparative scale, samples of about 0.25–0.5 ml and 96–99% purity were obtained from mixtures of isomers with similar boiling points, such as II and III (see EXPERIMENTAL).

EXPERIMENTAL

Apparatus and procedure

Gas–liquid partition chromatography was carried out in a Perkin Elmer 154 A Fractometer, essentially according to the procedure employed previously[8]. The temperature was 30° and the flow rate of helium 36 ml/min and 42 ml/min for the C_6 and the C_7 isomers, respectively. Toluene was taken as the internal standard, since it was found to be more suitable than ethylbenzene used for the methylcyclohexene isomers. The R_V of ethylbenzene relative to that of toluene is 1.54.

Materials

1-Methylcyclopentene (I) was prepared by dehydration of 1-methylcyclopentanol with dilute H_2SO_4. The olefin, after distillation through a Piros Glover spinning band micro column, had a purity of approximately 99% as determined by G.L.P.C., and b.p. 75.6° (760 mm); n_D^{20} 1.4330, (reported[11] 75.8°; 1.4330). The absorption in the infrared was found to be in full agreement with the spectrum published by KOCHLOEFL *et al.*[7].

3- and 4-Methylcyclopentene (II and III). 3-Methylcyclopentanone was prepared by oxidation of 4-methylcyclohexanol and thermal decomposition of the resulting β-methyladipic acid[12]. Hydrogenation of the product under pressure in the presence of Raney nickel gave 3-methylcyclopentanol, free of ketone. B.p. 149–150° (757 mm); n_D^{27} 1.4403, (reported[13] 149–150° (750 mm)). 4 g of the alcohol were converted into the acetate, which was then pyrolysed at 550° and about 10 sec contact time over Pyrex beads in a tube of 8 mm diameter and 30 cm length. The product consisted of 60% of 3-methylcyclopentene and 40% of the 4-methyl isomer. Isolation of the isomers by fractional distillation of the reaction mixture was not possible. However, about 0.25 ml of each component was prepared in a state of 96–97% purity by gas–liquid chromatography of successive small portions of the mixture on a column of 10 mm diameter and 2 m length, and a saturated silver nitrate–glycol solution as the stationary phase. The isomers were identified by comparison of their absorption curves in the infrared with the spectra given by KOCHLOEFL *et al.*[7]. The chromatographic data for 3- and 4-methylcyclopentene were obtained by using the mixture of the two isomers.

3-Ethylcyclopentene was synthesised by condensation of ethylmagnesium bromide with 3-bromocyclopentene. The other *C_7 cyclopentenes* and *methylenecyclopentane* were obtained from the corresponding alcohols or acetates. Full experimental details will be given elsewhere. The purity of these compounds, as determined by G.L.P.C., was above 99%, except for 4-ethylcyclopentene which was 98.5% pure.

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SUMMARY

Gas–liquid partition chromatography of C_6 and C_7 alkylcyclopentenes and alkylidenecyclopentanes over a saturated silver nitrate–glycol solution has been studied. Mixtures of isomers, including those whose boiling points differ by only 0.1°, could be easily separated and analysed quantitatively.

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THE PROPERTIES OF PROTACTINIUM(V) IN ALKALINE SOLUTIONS

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In a preliminary note[1] we have reported that a soluble form of Pa(V) may be obtained by fusing ^{233}Pa tracer in NaOH and diluting with water. In such solutions Pa(V) was shown to move on filter paper when developed with N KOH and to migrate slowly toward the anode in paper electrophoresis with N KOH as electrolyte.

In this paper we wish to describe further work on the behaviour of Pa(V) in alkaline solutions.

EXPERIMENTAL

I. The preparation of solutions of Pa(V) in alkali

^{233}Pa tracer in 6 N HCl was evaporated in a microbeaker, a few pellets of NaOH or KOH were added and fused over a naked flame for a few minutes, cooled and diluted with water to yield a solution 5 N with respect to alkali. Such solutions usually contain a soluble fraction but also an insoluble activity. If the solution in HCl is taken to dryness and moistened with conc. HCl and again evaporated and this process repeated three times the insoluble compound does not form. It seems that during evaporation with 6 N HCl some radiocolloid is formed which does not react readily with NaOH. When evaporated repeatedly with conc. HCl this seems to be inhibited and presumably the Pa(V) is left in the beaker as a very thin layer on the surface which then reacts readily with fused NaOH.

When solutions which have been evaporated three times with conc. HCl are treated with aqueous 6 N KOH some transformation into a soluble form was also noted. Without this pretreatment as reported previously[1] no soluble fraction is obtained.

II. Paper chromatography

Our first evidence for the solubility of Pa(V) was found by chromatographing solutions prepared as in (I) on Whatman No. 1 paper (unwashed) with N NaOH or N KOH as solvent. The Pa(V) has here an R_F value of about 0.5. We then tried to obtain evidence on the mode of adsorption and stability of the protactinate ion by chromatographing with various concentrations of NaOH and KOH. However, no consistent results could be obtained further than that Pa(V) remains soluble under various conditions.

The studies with anion exchange resins (described below) then indicated that the Pa is present as a polyvalent anion. In this case the impurities of the paper (Ca, Mg,

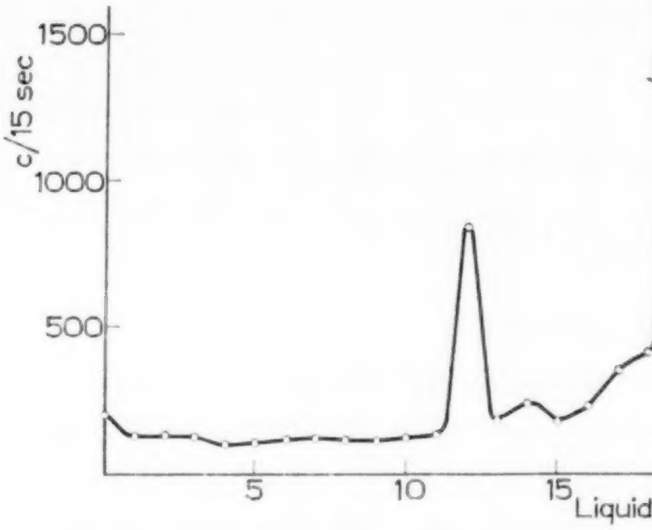


Fig. 1

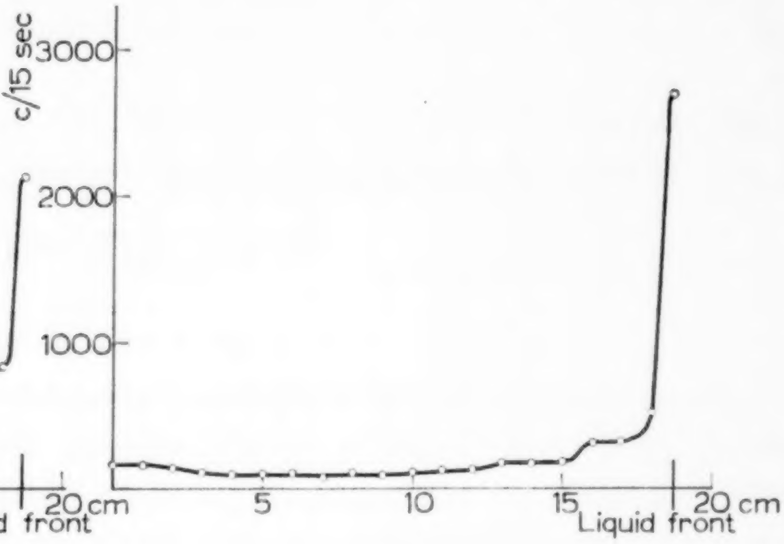


Fig. 2

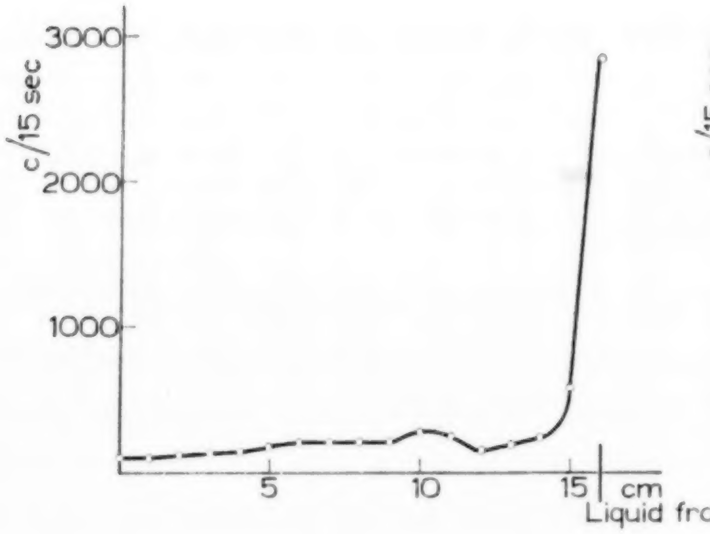
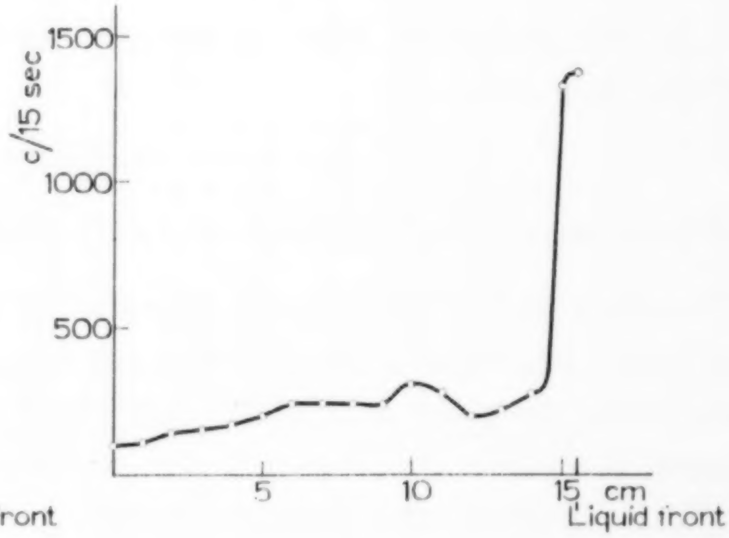


Fig. 3



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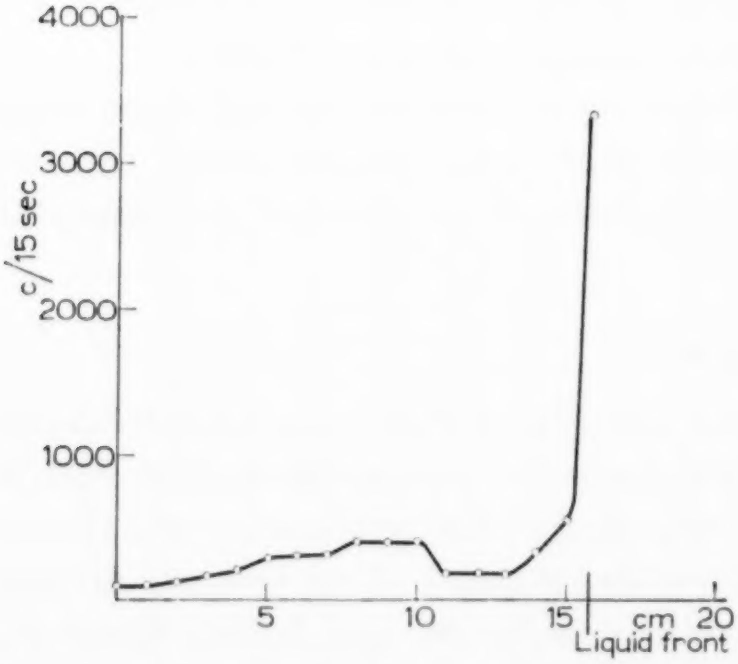

Fig. 5

Figs. 1–5. Paper chromatograms of protactinate developed on washed Whatman No. 1 paper with: (1) 0.1 *N* KOH. (2) 0.5 *N* KOH. (3) 1 *N* KOH. (4) 2 *N* KOH. (5) 3 *N* KOH.

Fe and Cu) would have to be removed by washing with HCl and water to prevent precipitation of the protactinate or a complexing agent such as ethylenediaminetetraacetic acid (EDTA) added to complex these impurities.

Chromatograms on washed paper are shown in Figs. 1–5. The protactinate moves with the liquid front and not as on unwashed paper with an R_F 0.5 and is stable down to 0.1 *N* KOH, the lowest concentration of alkali tried as developer. In 0.1 *N* KOH two peaks of activity are however formed. On unwashed paper with alkali solutions containing 1% EDTA the elution is not as complete. In 2 *N* KOH most of the Pa moves rather fast but in lower concentrations appreciable amounts stay at the start. Thus EDTA does not complex all metals efficiently at lower concentrations of KOH.

III. Anion exchange of protactinate

Protactinate solutions in KOH from 0.2 to 2 *N* were equilibrated with weighed amounts of Dowex-2. Good results are only obtained when all solutions are freshly prepared and kept in stoppered bottles as absorption of CO_2 from the atmosphere may alter the equilibria considerably. 1 g lots of Dowex-2 were converted to the OH form by washing three times with 2 *N* KOH and three times with distilled water. The solution of protactinate in the required concentration of KOH was then added and shaken for two hours to reach equilibrium, a portion withdrawn and its activity determined, and the amount adsorbed calculated from the loss of activity. The equilibrium constant K_d was then calculated and its logarithm plotted against log OH^- as shown in Fig. 6.

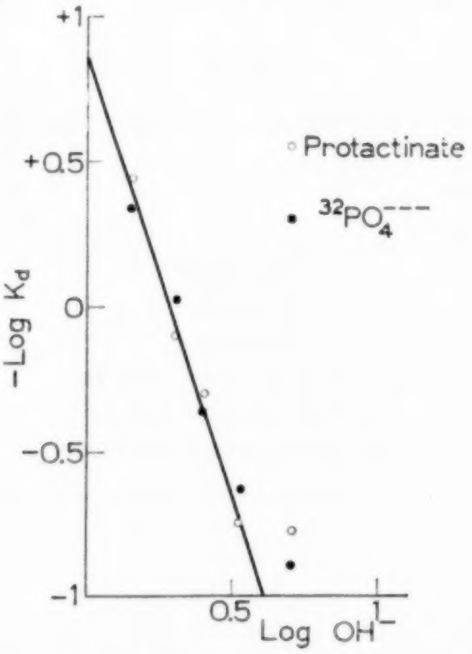

Fig. 6. Anion exchange adsorption of protactinate and tracer $^{32}PO_4^{-3}$ in KOH of varying concentrations. —log K_d is plotted against log OH^- of the solution.

$$K_d = \frac{\text{amount adsorbed}}{\text{amount in solution}} \times \frac{\text{volume of solution}}{\text{mass of resin}}$$

In this equilibrium between OH^-, a monovalent ion, and protactinate the tangent of the line obtained when log K_d is plotted against log OH^- should indicate the valency of the protactinate ion. The tangent in Fig. 6 is 3.1 thus indicating that the protactinate, if present as a single anion and not a mixture of several anions of different valencies, would have a valency of three. In order to check our experimental method and calculations we also carried out identical equilibrium experiments with tracer $^{32}PO_4^{-3}$. As also shown on Fig. 6, phosphate gives results identical to protactinate.

IV. Adsorption on glass

In order to prevent an error due to adsorption on the walls of the test tubes used in the above ion-exchange experiments, a "blank" experiment, *i.e.* one without the resin, was carried out for each concentration of KOH. The adsorption on glass was found to be negligible and below the accuracy of our activity measurements.

V. Solvent extraction

Solutions of protactinate in *N*, 2 *N* and 3 *N* KOH were shaken with equal volumes of butanol and methyl propyl ketone. The partition coefficients were determined by measuring the activity in both the aqueous and the solvent phase. Table I shows that the amount extracted is very small in all cases.

TABLE I

SOLVENT EXTRACTION OF PROTACTINATE

Normality of KOH	*Solvent*	*% extracted (approximate)*
1	Butanol	2
2	Butanol	5
3	Butanol	5
1	Methyl propyl ketone	7
2	Methyl propyl ketone	4
3	Methyl propyl ketone	4

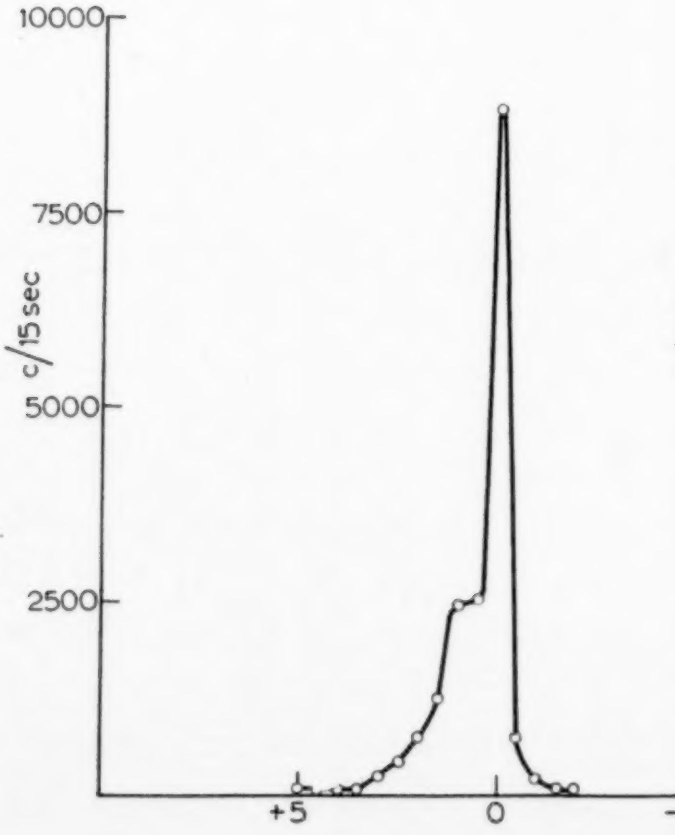

Fig. 7

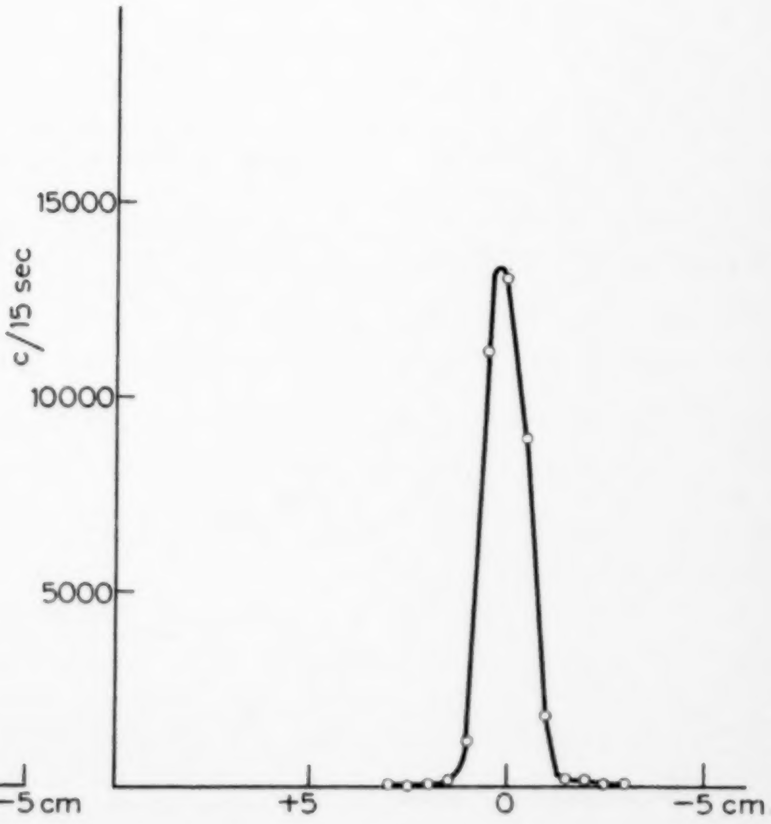

Fig. 8

References p. 417.

VI. Precipitation with metal ions

In our paper chromatographic work we have reported that the retention of protactinate was due to metal impurities in the filter paper. We thus devised a simple method for determining when protactinate is precipitated or coprecipitated, by placing a drop

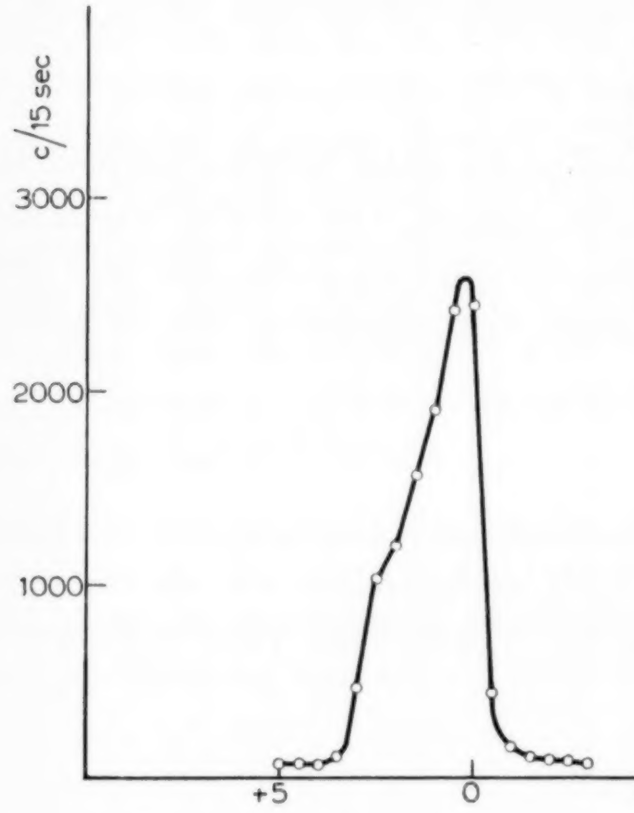

Fig. 9

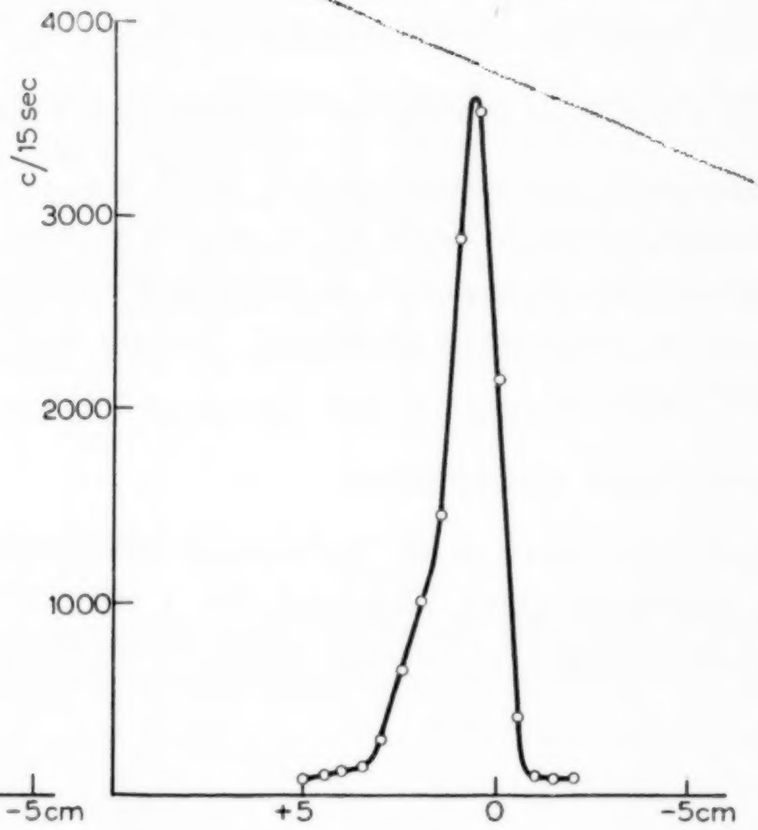

Fig. 10

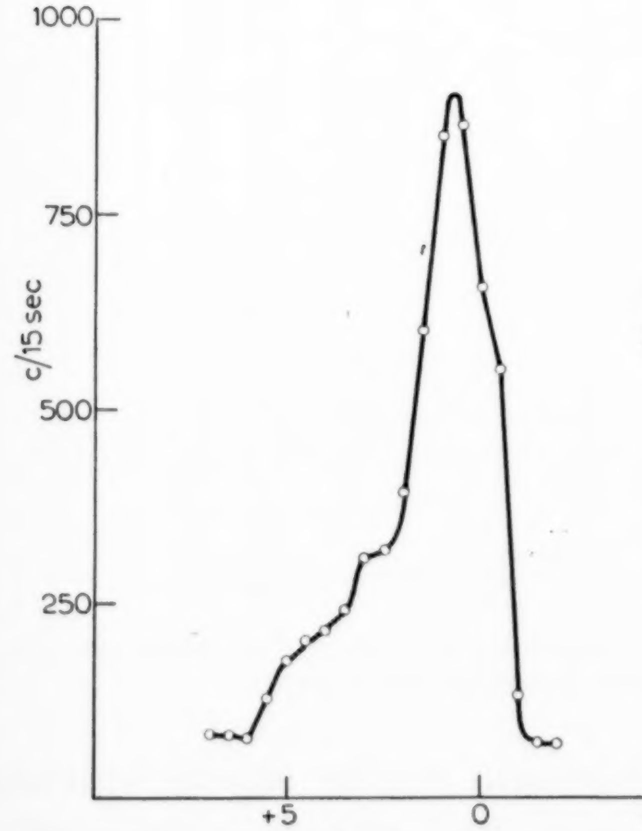

Fig. 11

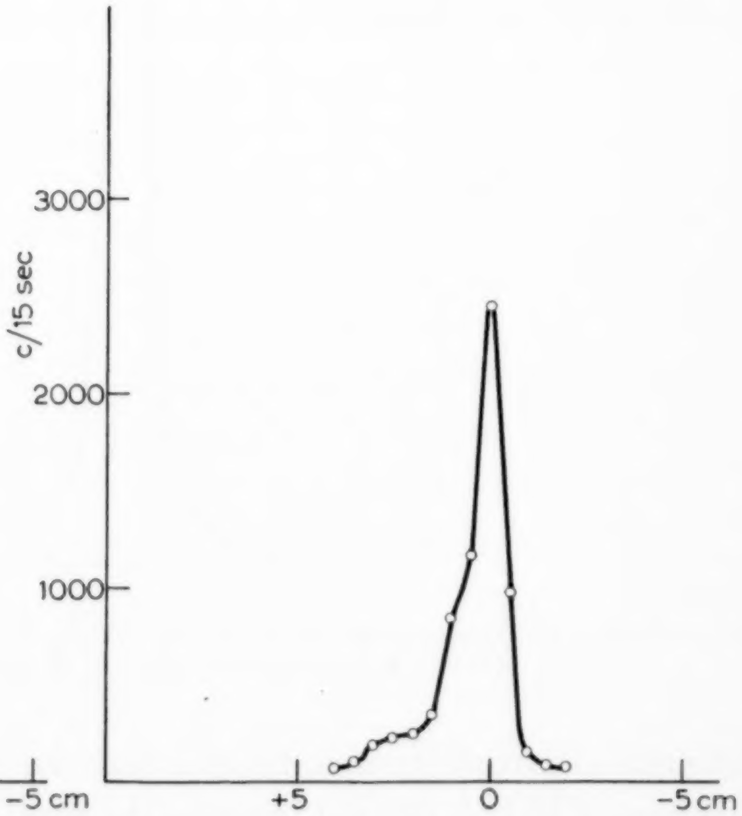

Fig. 12

Figs. 7–12. Paper electropherograms of protactinate. Conditions: (7) 1 hour, 250 V, electrolyte 1 *N* KOH, washed Whatman No. 1 paper, glass plates. (8) as (7) with Whatman glass fibre paper instead of No. 1 cellulose paper. (9) as (7), electrolyte: 1 *N* KOH containing 1% EDTA. (10) as (9) with plexiglass plates replacing the glass plates. (11) as (10), electrolyte: 0.1 *N* KOH containing 1% EDTA. (12) as (7), electrolyte: 0.1 *N* KOH, 1% EDTA and 1% K_3PO_4.

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of protactinate followed by a drop of the metal ion solution on washed paper and developing with *N* KOH. When the activity is retained on the point of application or retarded this must be due to insolubilisation by the metal ion.

Some, but relatively little, precipitation occurred with Mg, Ti(IV) and Zr(IV). Almost complete retention at the start was obtained with La, Fe(III), Sr(II) and Tl(I). Complete precipitation occurred with Ba, Ca and Mn (which forms MnO_2).

VII. Stability of protactinate solutions to heating

Solutions of protactinate in 6 *N* KOH were placed on the water bath for one hour and developed with *N* KOH on washed Whatman No. 1 paper. The activity was always found on the liquid front, sometimes divided into two adjacent peaks (as is also often found in non-heated solutions), but no indication of precipitation due to heating could be noted.

VIII. Paper electrophoresis

As already shown in our preliminary note, protactinate moves somewhat to the anode on unwashed paper Whatman No. 1 with *N* KOH as electrolyte. On washed paper the movement is of the same order, *i.e.* the peak moves at the most 5 mm to the anode.

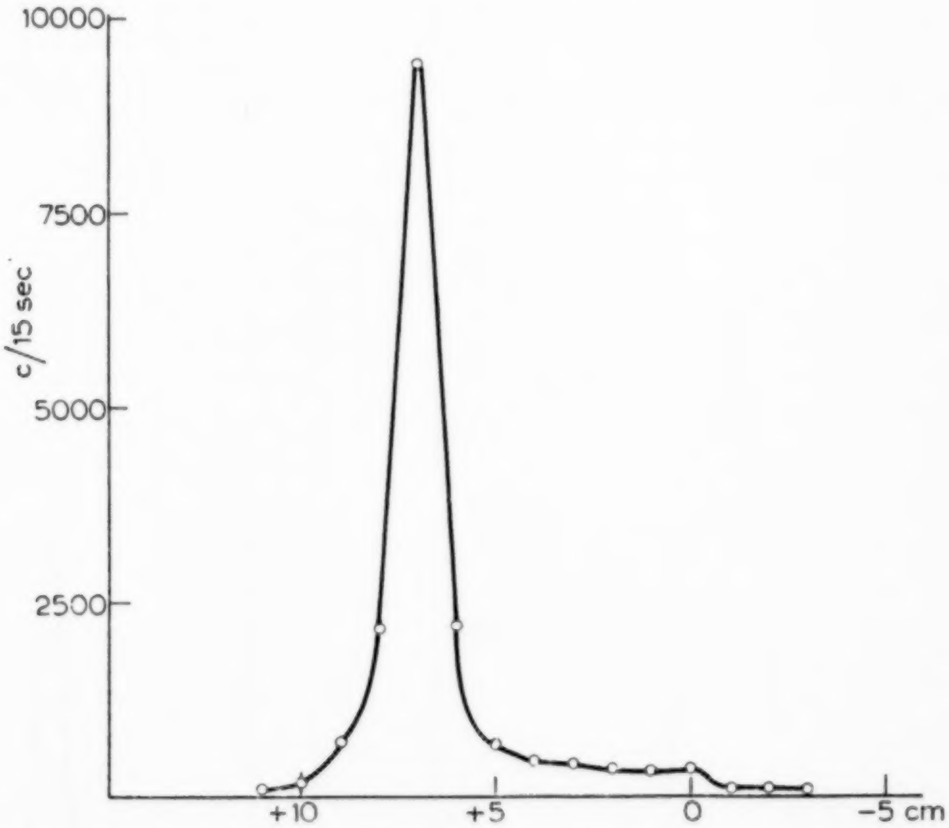

Fig. 13. Paper electropherogram of tracer $^{32}PO_4^{-3}$. Conditions: 1 hour, 250 V, electrolyte 0.1 *N* KOH containing 1% EDTA, washed Whatman No. 1 paper.

Prolonged electrophoresis indicated that the movement does not increase with time. Additions of EDTA did not increase the movement either, nor did electrophoresis on glass paper or on filter paper with perspex plates replacing the usual glass plates. Figs. 7–13 show the activity curves obtained. They also show the movement of $^{32}PO_4^{-3}$ tracer and the movement of protactinate in presence of 1% K_3PO_4 to inhibit adsorption (if any).

CONCLUSION

We may summarise the properties of protactinate solutions at tracer levels in alkali as follows: It is readily soluble in aqueous alkali, does not hydrolyse in 0.1 *N* KOH and is not decomposed when heated in solution to 100°. It is not extracted to any extent into butanol or methyl propyl ketone, nor adsorbed strongly on glass. It is precipitated by Ba, Sr, Ca, Tl(I) and coprecipitated with MnO_2, La and Fe(III). On Dowex-2 adsorption occurs and the adsorption equilibrium indicates that protactinate has a charge of three. However, the slow movement in paper electrophoresis is incompatible with the high charge exhibited in ion exchange. Orthophosphate tracer under the same conditions moves with a much higher speed. It is thus not possible from these tracer experiments to suggest a probable structure for the protactinate ion. The slow electrophoretic movement may be caused by the existence of the protactinate in a polymeric form or to ion pair formation with K^+ ions, the latter being less likely than the former.

SUMMARY

The paper chromatography, paper electrophoresis, anion exchange, solvent extraction and precipitation reactions of tracer quantities of protactinate are described.

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ANALYSIS OF ADENINE POLYPHOSPHATES BY PAPER CHROMATOGRAPHY

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INTRODUCTION

The microanalytical determination of adenosine triphosphate and adenosine diphosphate is still a topic of discussion. Early methods still in use rely on the enzymic hydrolysis of phosphate groups from adenine polyphosphates[1]. Later methods have used firefly luminescence as a biological assay technique. Chemical methods are divided between those that estimate total adenine, such as that by Pirwitz *et al.*[2], which relies on the colorimetric estimation of adenine, and the separation of adenine polyphosphates by paper chromatography and their subsequent analysis by phosphate estimation. Such a technique was first proposed by Hanes and Isherwood[3].

In this paper it is proposed to discuss a technique utilizing preliminary paper chromatographic separations followed by the analysis of the adenine by absorption at λ_{max} 260. This technique has been used in this Department for the estimation of ATP[*] and ADP in large numbers of toad sartorii.

EXPERIMENTAL

The treatment of sartorius muscles which were taken from the toad, *Bufo marinus*, was essentially similar to that previously reported by Simon *et al.*[4]. Special care was taken in blotting each muscle as contractions can occur. This affects the (resting) creatine phosphate level although the ATP content remains essentially unchanged[5]. At the conclusion of each experiment the muscles were frozen in liquid air. Each was then homogenized in a chilled glass homogenizer with 2 ml of 5% trichloracetic acid solution. The homogenate was centrifuged at 2° and the supernatant analysed as follows:

(a) Neat supernatant was analysed for ATP and ADP.

(b) Dilutions were estimated for creatine and creatine phosphate by a similar method to that employed by Ennor and Rosenberg[6]. The cations, sodium, potassium and lithium, rubidium and caesium (if any) were measured by flame photometry[7]. 50 ml of each extract were spotted onto a Whatman No. 1 Chromatography Paper so that a pair of spots and a blank were run on the *same* muscle pair.

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[*] Abbreviations used: ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate.

Each chromatogram was run by a similar technique to that of KREBS AND HEMS[8], using isopropyl ether–90% formic acid (3:2) for 5 hours at 20° and then drying the paper in a draft cupboard at room temperature. The paper was then cut 1½ in. above the starting line and the chromatogram developed with isobutyric acid, *M* ammonia, with a trace of 0.1 *M* EDTA solution (100:60:1.6), overnight. The paper was dried for 2 hours at room temperature and photographed in the ultraviolet by a contact method similar to that suggested by MARKHAM AND SMITH[9, 10]. A B.G.E. Germicidal lamp in an industrial metal housing, fitted with a wooden front, provided the illumination and "Kodagraph" Paper (Extra Thin) was used. The photos clearly show the presence of ATP, ADP and AMP (if significant). The paper was then cut and the adenine phosphate eluted with 6 ml of water. The adenine was estimated in a Beckman Spectrophotometer at $\lambda_{\max}$ 260 mμ[11], using a photomultiplier attachment to increase the sensitivity of the instrument. Standard ATP samples (Sigma) of 0.020, 0.040 and 0.060 μM were also run with each determination.

RESULTS

In 64 experiments the average value obtained for muscles soaked for 4 hours in normal Ringer solution is 5.9 mmoles/kg for ATP and 2.5 mmoles/kg for ADP. This includes substantial variations in season which are correlated with creatine phosphate in the results. Normal ATP values range from about 3–8 mmoles/kg of soaked muscle. Muscles soaked in various abnormal ionic Ringer solutions and solutions of normal Ringer with added metabolic inhibitors also show good correlation. These results are given in another paper. The values correlate well with those recorded by HARRIS[13]: ATP + ADP 7.7 – 13.8 mequiv./kg.

DISCUSSION

It was necessary to design a method which could be used on a large number of muscles, which gave comparable results and where the rest of the analysis could be conducted on the same extract.

The original method tried was that of EGGLESTON AND HEMS[8], but we found that it had the following disadvantages.

(i) The separation of creatine phosphate and inorganic phosphate was not clear and besides, creatine phosphate is unstable in the acid solvent used.

(ii) The subsequent analysis was very time-consuming as the total incineration of each paper required watching and a minimum of 33 incinerations would have had to be carried out for each experiment, each of which taking 10 minutes.

(iii) The subsequent analysis of phosphate by the method of BERENBLUM AND CHAIN[14] was also laborious and the reagents used were most expensive.

(iv) It was found that most u.v. absorbing substances were removed from the paper by the first solvent and subsequent chromatography with versene forced all the remaining contaminants to the solvent front. It was therefore considered unnecessary to wash papers.

(v) It is doubtful if the possible extra accuracy of the ATP level is warranted in the experiment, as the variation of ATP level in toad is very variable and statistical methods used in the experiment required only the variation in pairs of muscles. The photography with ultraviolet light was first suggested by MARKHAM AND SMITH[9, 10]. In their method a Hanovia "Homesun" lamp was used, fitted with filters. This was not available in the laboratory and it was found that the British General Electric Germicidal lamp could be utilized for the same purpose.

The use of blue print copying paper[15] has been tested using "Ozaprint". This has the advantage of not requiring a darkroom, provided no direct sunlight falls on the paper. Against this, it requires an exposure time of 30 minutes compared with 15 seconds needed for contact paper. A paper scanner attachment for the Beckman Spectrophotometer[16] was not available so that elution methods had to be used. An elution with water by a capillary method[17] was found to require overnight elution. An apparatus treating up to 36 paper strips simultaneously was difficult to design. It was found that if the chromatogram was macerated in water with a plastic flanged rod in a centrifuge tube, a clear supernatant in a known volume of water was readily obtainable by centrifuging the mixture for 40 minutes at 1150 *g*. The total time for elution of 16 samples was reduced to 1 hour. Merely soaking for 1 hour[11] produced variable results. The sample was analysed at λ_{max} 260 mμ in a Beckman Spectrophotometer equipped with a photomultiplier attachment. It was found that under these circumstances, both ATP and ADP had a maximum peak of λ_{max} 258.5 mμ.

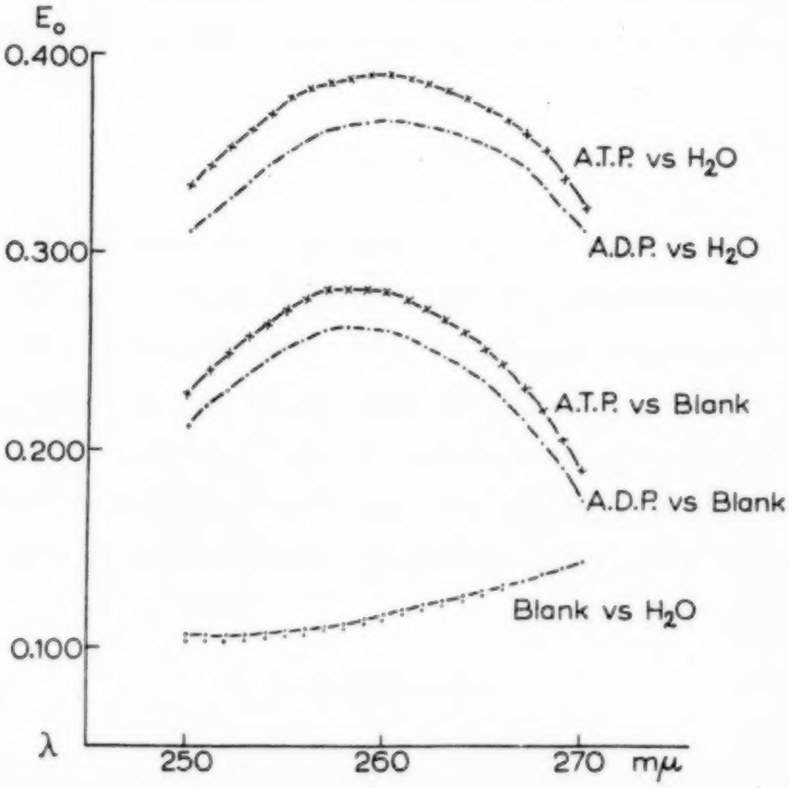

Fig. 1. Adenine spectrum.

These are shown in the graph (Fig. 1). The values in both standard and muscle extract gave symmetrical curves when E_0 was plotted against the wave length. Elution with buffered solution at pH 2 (0.1 M HCl–KCl) gave similar E_0 readings although it was necessary to adjust the blank zero slightly.

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Choice of blank

Much of the success of the method depends on the choice of the blank. To reduce "blank" error, chromatograms 6 in. wide were divided by pencil lines into three strips. A blank was first run. The result of this can be seen in the diagram (Fig. 2). It will be seen that variation is least when horizontal blanks are used.

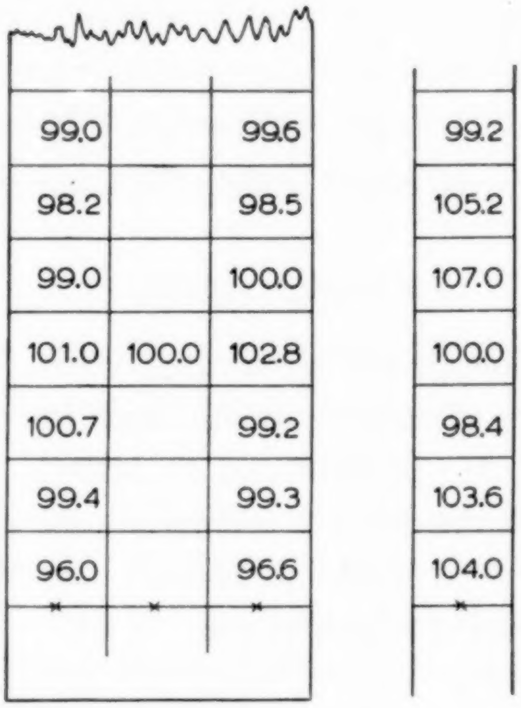

Fig. 2. Transmittance at 260 mμ. Blank: Whatman No. 1 paper.

Advantages of the method

The greatest single advantage of the method is speed. It is possible to complete assay of 8 muscles for ATP and ADP in 24 hours, thus minimizing breakdown of ATP. The apparatus specifically designed for this method is very inexpensive. More samples can easily be analysed by the provision of more chromotanks. A similar procedure can also be used for other microanalyses. It has recently been used for determination of strychnine in *Nux vomica*[18].

Limitations and precautions

The value is relative only and depends on a number of factors.

(a) The isobutyric acid is very difficult to remove from the paper. This means that if the method is not rigidly adhered to, the absolute result may be in error, although the blank will cancel most of the effect. It may be desirable to use more easily removable solvents such as those suggested by GERLACH *et al.*[19]. These have not been tested fully in the laboratory.

(b) If the chromatograms are not placed squarely into the solvent, the spots will not run horizontally and "blank" errors are usually produced.

(c) Prior treatment of the muscle can cause a variation in the ATP content of the muscle and all the work with animal material should be done as far as possible by one analyst if comparable results are required.

(d) The solutions do deteriorate and the chromatograms should therefore be run as soon as possible after the end of the experiment. In no case should separation be delayed for more than 24 hours, as significant breakdown occurs. Once the separation has taken place the chromatograms can be stored for a few days as the analysis is based on the adenine content.

Contamination can easily occur as many contaminants could absorb in the ultraviolet. It is necessary to observe strictest cleanliness both in the apparatus, which is specially washed with chromic acid and rinsed with distilled water, and in the laboratory generally.

ACKNOWLEDGEMENTS

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He also wishes to thank the National Health and Medical Research Council of Australia, who provided a grant for this work.

SUMMARY

A method for the microanalysis of adenine polyphosphates in toad muscles has been developed. This is based on the preliminary separation of the constituents by paper chromatography followed by the analysis of adenine by absorption at λ_{max} 260 mμ.

With this technique, it is possible to do a complete assay of eight muscles for ATP and ADP in twenty-four hours.

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ON THE RELATION BETWEEN SIDE CHAIN LENGTH AND R_M VALUE

A

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All the investigations concerning the partition chromatography of homologous series start from MARTIN's theoretical suppositions[1] about an additive influence of functional group increments on the values of the partition coefficients. BATE-SMITH AND WESTALL[2] confirmed the validity of these suppositions for hydroxy, carboxy and methylene group increments, and at the same time deduced a linear relation between the number of these groups and the R_M value. LONG[3] proved that this relation was roughly linear for methylene group increments in the homologous series of carboxylic acids. On the other hand, some other authors such as ISHERWOOD AND HANES[4] and KALBE[5] showed that for mono- and dicarboxylic acids the linear relation between R_M values and the number of carbon atoms is valid in a limited range only.

In the course of our studies on the relation between the structure of organic substances and their chromatographic behaviour, we have established earlier[6] that a substantial difference exists according to whether the increase by the homologous increment takes place in the substituent attached to an aromatic nucleus or to a side chain. When a methyl group is introduced into the aromatic nucleus, the formula deduced by BATE-SMITH is valid. On the other hand, if a methylene group is introduced into the side chain, the relation between the R_M value and the number of carbon atoms is linear practically for the first three members only; the R_M value increments for further members, however, decrease with the length of the chain (Fig. 1).

The relation between the number of hydroxy and carboxy groups and the R_M

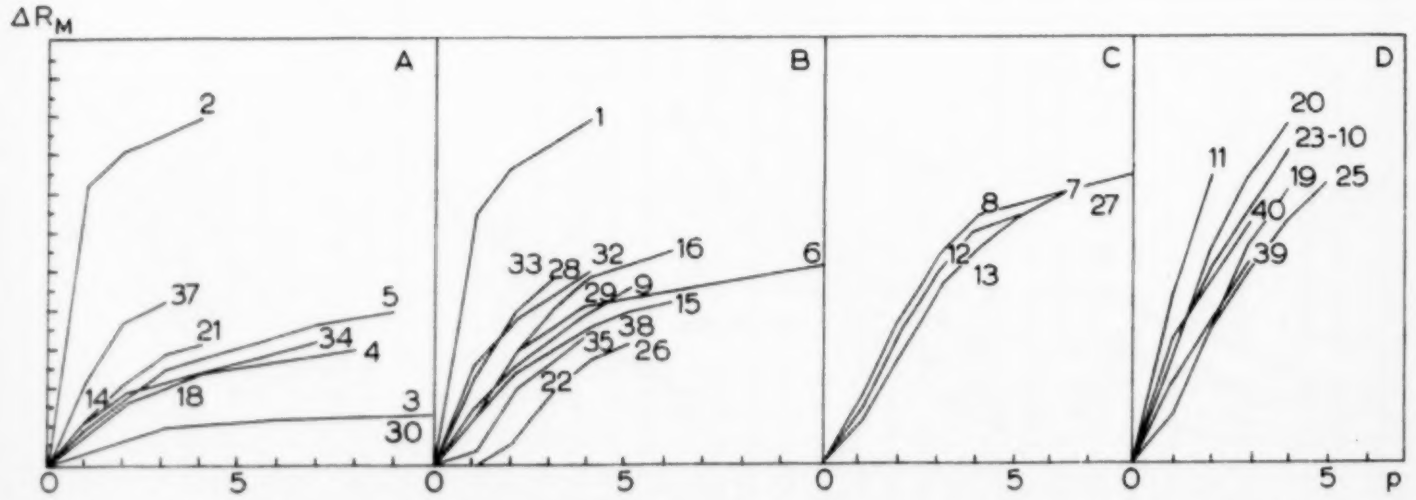

Fig. 1. A, B, C, D. Relation between the number of carbon atoms of a side chain and the difference in R_M values. (p represents the difference in the number of carbon atoms.) The numbers correspond to those in Table I.

value cannot be estimated in more than about three members of homologous series, because the presence of such groups causes an appreciable difference in R_M values. Where more of the groups are present, further members of the series are found in an inconvenient R_M range, *i.e.* in close proximity to the start or the front.

For the above reason, in this investigation we have studied only the homologous increment —CH$_2$— which can be followed in a sufficient number of members of homologous series. In this case, increase of the number of methylene groups by one link results in only slight changes in the R_M values. We selected from the literature the R_M values of a wide variety of organic compounds forming comparatively long homologous series.

DEDUCTION OF THE RELATION BETWEEN THE HOMOLOGOUS INCREMENT AND THE R_M VALUES

The relation between the ΔR_M value and the homologous increment for a number of homologous series derived from the literature is plotted in a linear scale in Fig. 1. It is obvious from this figure that the relation is not linear in any of the quoted examples. The logarithmic relation makes simpler and more exact expressions possible. This is evident from Fig. 2, where the given dependence was plotted logarithmically. Thus, the following equations can be proposed:

$$R_{Mp} = k \cdot \log p + R_{M1} \tag{1}$$

$$k = \frac{R_{Mp} - R_{Mq}}{\log (p/q)} \tag{2}$$

where k is constant for a given homologous series and given chromatographic conditions, and R_{Mx} corresponds to the x-th member of a homologous series after the

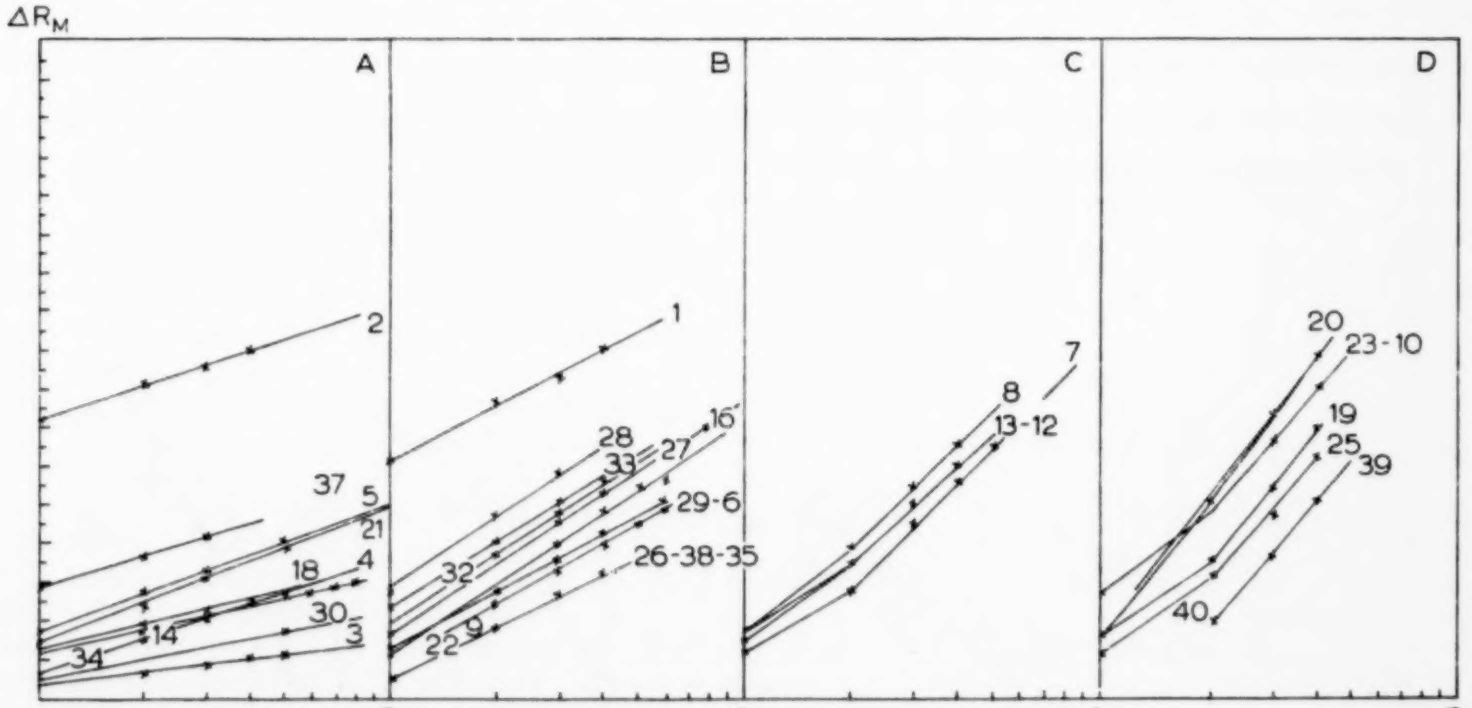

Fig. 2. A, B, C, D. Relation between the number of carbon atoms of a side chain and the difference in R_M values. (p values are given in the logarithmic scale.) The numbers correspond to those in Table I.

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TABLE I

LIST OF CONSTANTS k AND (R_{Mp}—R_{Mo}) VALUES

No.		*k*	R_{Mp}—R_{Mo}	*Type*	*Stationary phase*	*Mobile phase*	*Reference*
15	Alcohols as diphenates	0.76	0.23	A–B		isoamyl alcohol–aq. ammonia–water	7
16	Alcohols as nitrophthalates	1.28	0.18	B			7
5	Alcohols as dinitrobenzoates	0.72	0.33	A		heptane–methanol	8
27	Alcohols as dinitrobenzoates	1.06	0.47	B	dimethylformamide	decalin	11
28	Alcohols as dinitrobenzoates	1.14	0.58	B	dimethylformamide	cyclohexane	12
29	Alcohols as alkylisothiuronium salts	0.96	0.25	B		*n*-amyl alcohol–acetic acid–water	13
32	Alcohols as dinitrobenzoates	1.26	0.38	B		ethyl acetate–dioxane–water	9
33	Alcohols as alkoxydinitrobenzoates	1.12	0.50	B	dimethylformamide	cyclohexane	12
22	Ketones as rhodanines	1.06	0.14	B	dimethylformamide	chlorocyclohexane	14
23	Ketones as rhodanines	2.08	0.31	D	formamide	chlorocyclohexane	14
10	Ketones as rhodanines	2.08	0.30	D	formamide	tetrachloromethane	14
18	Ketones as thiosemicarbazones	0.44	0.25	A	dimethylformamide	chlorobenzene	15
19	Ketones as thiosemicarbazones	2.30	0.32	D	formamide	cyclohexane–butanol	15
20	Ketones as thiosemicarbazones	2.42	0.31	D	formamide	chlorobenzene	15
39	Ketones as *o*-nitrobenzaldehyde derivatives	2.00	—	D	acetamide	cyclohexane–butanol	16
40	Ketones as *o*-nitrobenzaldehyde derivatives	2.02	—	D	acetamide	cyclohexane	16
21	Aldehydes as dinitrophenyl-hydrazones	0.72	0.22	A		light petrol–methanol–ethyl acetate	17
13	Aldehydes as 2,4-dinitrophenyl-hydrazones	1.68	0.18	C	methanol	heptane	18
3	Aldehydes as 2,4-dinitrophenyl-hydrazones	0.48	0.06	A		ethyl ether–light petrol	19
37	Aldehydes as 2,4-dinitrophenyl-hydrazones	0.60	0.56	A	dimethylformamide	cyclohexane	20
26	Amines as hydrochlorides	0.92	0.08	B		*n*-butanol–acetic acid–water	21
9	Amines as hydrochlorides	0.86	0.14	B		*m*-cresol–acetic acid–water	22
38	Indolylcarboxylic acids	0.90	0.12	B		butanol–aqueous ammonia–water	23
4	Aliphatic acids as hydroxamic acids	0.42	0.24	A		phenol	24
6	Aliphatic acids as hydroxamic acids	1.00	0.24	B		*n*-butanol	24
35	Aliphatic acids as hydroxamic acids	0.96	0.08	B		butanol–dimethylformamide–water	25
7	Aliphatic acids as hydroxamic acids	1.72	0.06	C		amyl alcohol–acetic acid–water	26
8	Aliphatic acids as hydroxamic acids	1.76	0.32	C		methyl ethyl ketone	26
25	Aliphatic acids as hydrazides	1.96	0.12	D		isoamyl alcohol–collidine–water	27
14	Ketoacids	0.50	0.24	A		butanol–formic acid–water	28
30	Ketoacids (dicarboxylic)	0.42	0.06	A		aqueous ammonia	16
1	Aliphatic dicarboxylic acids	0.88	1.26	B		*tert*.-butanol–benzyl alcohol–water–formic acid	29
2	Aliphatic dicarboxylic acids	0.56	1.44	A		iso-octane–ethanol–acetone–formic acid	29
11	Aliphatic dicarboxylic acids	2.00	0.86	D		acetone–water–aqueous ammonia	30
34	Aliphatic dicarboxylic acids	0.60	0.14	A		ethanol–water	31
41	Aliphatic dicarboxylic acids	2.60				butyl acetate–water	33
12	Phenols as aryloxyacetic acids	1.68	0.18	C		isoamyl alcohol–aqueous ammonia–water	32

fundamental member. From the chromatographic point of view, the *k* value is a criterion of the partitioning power of the system used.

We computed the *k* value for a number of homologous series and compiled the calculated results in Table I together with the $(R_{Mp}—R_{Mo})$ values which make plotting easier than the R_{Mp} values alone. Comparing the various *k* values, we find that it is possible to divide them roughly into four groups (see Fig. 2 A, B, C, D), in which this value depends substantially on the properties of the stationary and mobile phases only, the character of the chromatographed substance being of slight significance. It is interesting that the highest *k* values are found in solvent systems where the difference between the dielectric constants of the stationary and mobile phases is greatest (*e.g.* formamide–cyclohexane). The energy of possible intermolecular hydrogen bonds between the chromatographed substance and the solvent system seems to be decisive in this case. Therefore, the *k* values do not follow the statistical distribution, but show certain quantization. For example, the *k* value for a mobile phase with dimethylformamide as the stationary phase is approximately half that for the same mobile phase with formamide or acetamide as the stationary phase. Fig. 3 showing the statistical distribution of the *k* values clearly demonstrates that these values are arranged into several groups which differ from each other by about $\Delta R_M = 0.48$. Only the third group departs from this rule, probably owing to the small number of examples in this group. The value $\Delta R_M = 0.48$, however, corresponds to the change of R_M value caused by one intermolecular hydrogen bond of the type O–H···O[10].

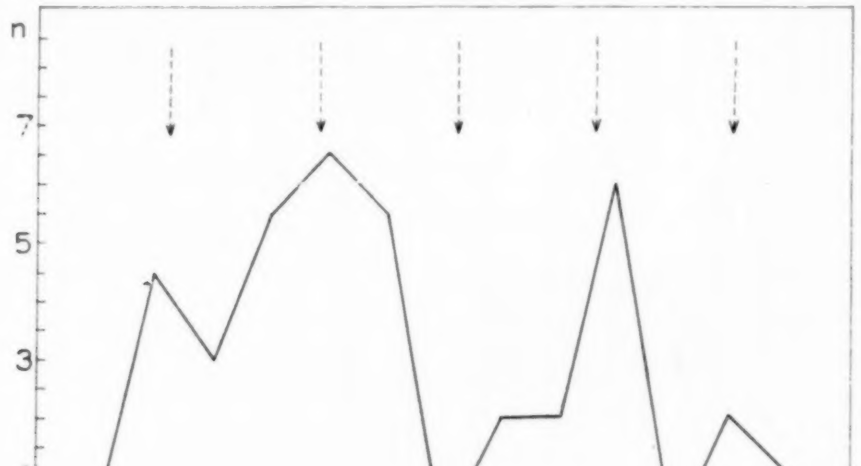

Fig. 3. The relation between the frequency *n* in an interval of $(K — 0.1; K + 0.1)$ and the *k* value.

The $(R_{Mp}—R_{Mo})$ values are probably influenced by the number of polar groups in the molecule, and increase with the number of these groups (*e.g.*, in the case of dicarboxylic acids).

The number of factors influencing the values discussed, however, is very large. Thus, it is hardly possible to draw accurate conclusions, since the range of experimental data is not very wide. In spite of this, we make the supposition that the relation between the number of carbon atoms in the side chain of the molecules and the R_M value is not linear but logarithmic.

References p. 427.

SUMMARY

A study was made of the R_M values, given in the literature, of a wide variety of organic compounds forming homologous series. An equation is proposed in which the R_M value is related to the number of carbon atoms in the members of the homologous series.

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SHORT COMMUNICATIONS

Adaptation of Mayer's reagent as a spray reagent for detecting alkaloids on paper chromatograms

Although Mayer's reagent[1] is widely used for precipitating alkaloids, the reaction involves no color change and therefore cannot be easily adapted to the detection of alkaloidal spots on paper chromatograms. For example, this reagent was not included in the thorough study by MUNIER AND MACHEBOEUF[2], while TSCHESCHE AND PETERSEN[3] developed a long and involved procedure for detecting alkaloids with Mayer's reagent.

While preparing the reagent by adding KI to $HgCl_2$, we noticed that a red HgI_2 precipitate formed first, which redissolved on addition of excess KI. This observation led to the development of the following procedure:

The dried paper chromatogram is sprayed with a 1% (w/v) solution of $HgCl_2$ in water. The strip is air dried and is then sprayed with a 0.5% (w/v) solution of KI. The alkaloids appear as white spots on a salmon background. Care should be taken not to spray KI excessively as it tends to decolorize the background.

Since this is essentially a Mayer's test, all alkaloids which form precipitates with the reagent are expected to respond to this spray reagent. Therefore, only a few alkaloids were tested, namely, brucine, nicotine, yohimbine, sparteine and reserpine. They all showed positive tests. A number of other nitrogen-containing compounds were also studied and quite a few gave positive tests. These include compounds containing a basic amino group or sulfur, *e.g.* ethanolamine, lysine, histidine, adenine, adenosine, adenylic acid, neomycin, thiamine, methionine and glutathione. Similar compounds neutral in nature are not detected by this reagent, such as urea, acetamide, glycine, alanine, uridine and caffeine.

It is therefore to be emphasized that this reagent is useful for detecting alkaloids but not to identify a spot as an alkaloid. However, the reagent might be used for the detection of other nitrogen-containing compounds as well.

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A sensitive probe for the Photovolt densitometer

Polycyclic hydrocarbons, separated into distinct spots on paper chromatograms, can be measured quantitatively by the visible fluorescence emitted under ultra-violet light. For this purpose we were using a Photovolt Densitometer type 501A, with ultra-violet source, and ultra-violet sensitive phototube. We considered that the sensitivity obtained with this arrangement was insufficient. Sensitivity could presumably have been improved by the purchase of a rather expensive photomultiplier photometer. Some preliminary experiments led us to believe that the requisite sensitivity could be obtained rather simply, and very inexpensively, using cadmium sulphide single crystal cells.

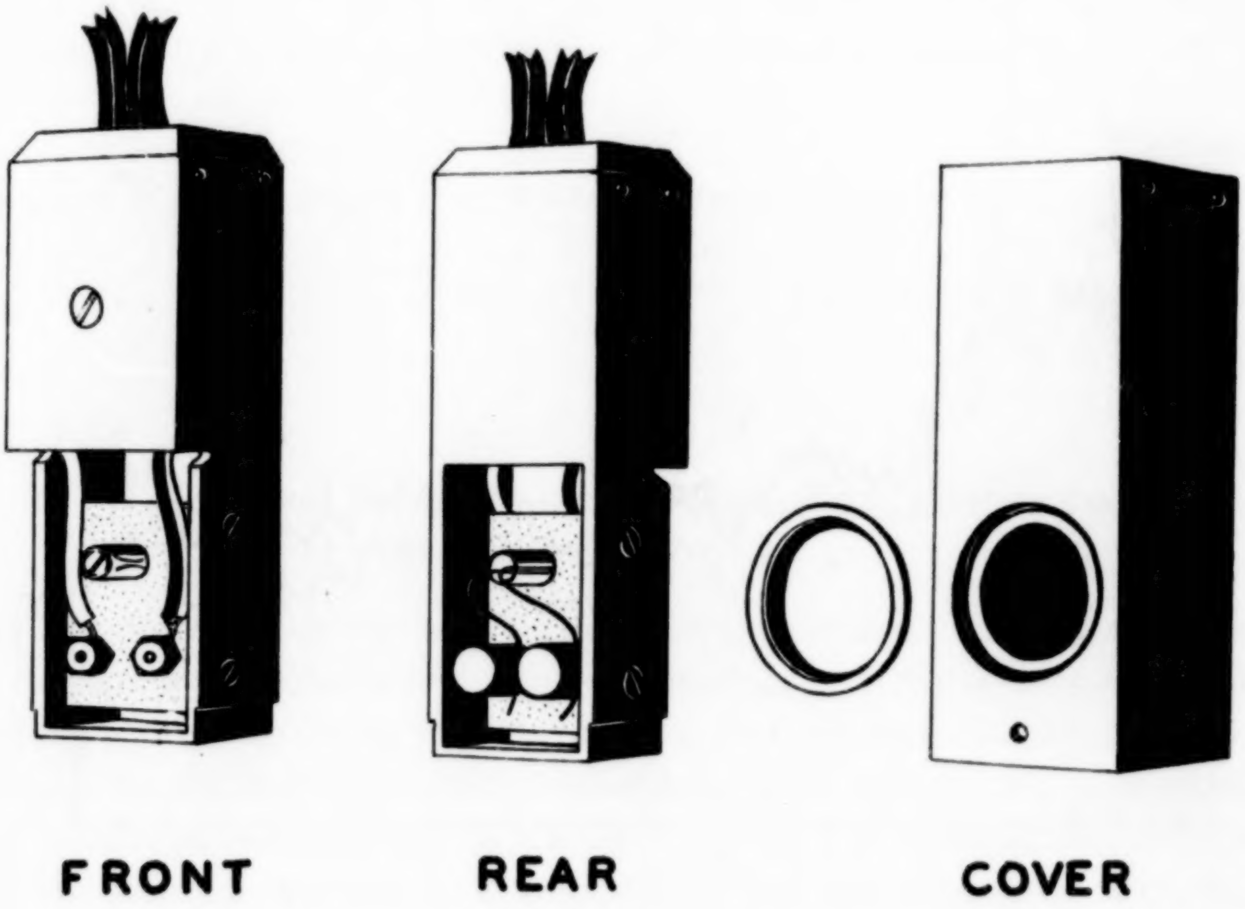

Fig. 1.

These preliminary experiments indicated that such a photocell (CL-2 of Clairex Computer N.Y.C., $ 3.50) showed an increase of about one hundred times for visible light over the original phototube. Accordingly, an adapter to hold this photocell was built for use with the Photovolt densitometer. The original probe was disassembled, and the phototube removed. By brazing aluminum angle stock together, an insert box was constructed fitted with covers on both ends, and cut out so that it would slip into the original cover without interfering with the filter holder ring. Inside the insert box the crystal cell was supported in a tight fitting hole drilled in a piece of plexiglass which also carried two binding posts. The photocell and the original cable were connected to the binding posts, the insert box slipped into the original rectangular housing, and the probe was ready to use (see Fig. 1).

Since the dark resistance of the crystal cell is much lower (1000 MΩ for a CL-2) it was necessary to adjust the coarse zero control, accessible on removing the top of the Photovolt photometer 501A.

In order to test the new probe a number of chromatograms of polycyclic hydrocarbons were run on a Photovolt type 520 densitometer, equipped with an ultraviolet source. The readings were recorded on a 10 mV Brown recorder. The same chromatogram was run using the Photovolt ultra-violet probe type B. Comparative tracings are shown in Fig. 2, for a chromatogram of 5 μg of pyrene. Microgram, and fractional microgram amounts also gave adequate response.

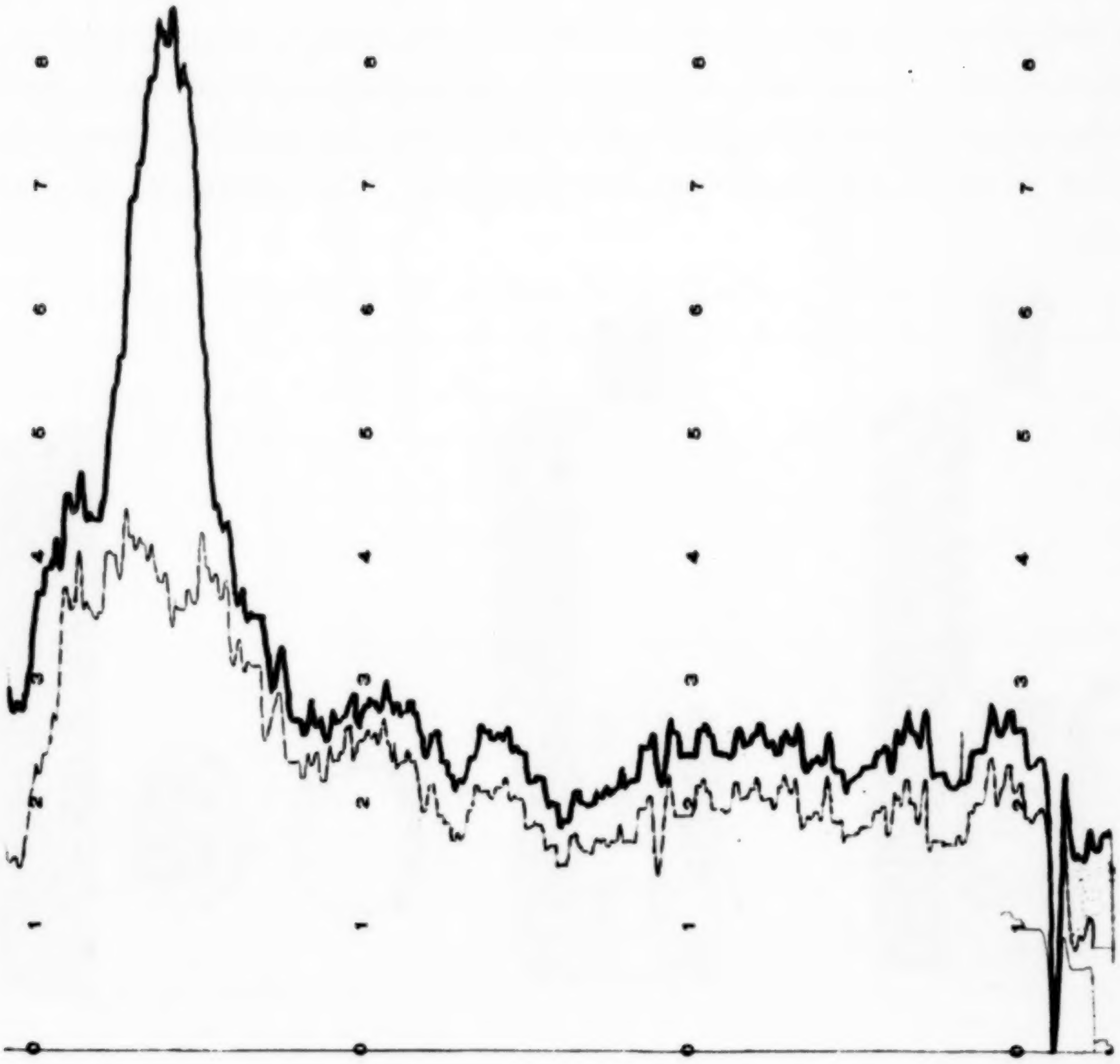

Fig. 2.

The Clairex cell gave sufficient sensitivity in range 1, whereas the phototube required the photometer range switch to be set at 2 or 3. By using the recommended ultra-violet filter No. 445 on the probe, the Clairex cell gave twice the sensitivity of the original phototube, although the CL-2 has a spectral peak at 520 mμ. Furthermore, we experienced less interference from line voltage fluctuations, when using the crystal photocell, due to the slower response time.

A further application was to use the new crystal probe to measure the light intensity on the screen of the Philips EM100 Electron Microscope. Here again the CL-2 showed its superiority over the ordinary phototube by a factor of approximately

200 on the meter reading. The phototube probe was practically useless at low intensities, whereas the crystal cell gave adequate readings well below the minimum intensity needed for focussing.

In our opinion, the sensitivity of a large number of photometric devices can be improved by substituting crystal photocells in place of phototubes provided:

(a) the lower internal resistance of the crystal cell can be accommodated;

(b) the longer response and decay time is not objectionable;

(c) the comparatively high temperature coefficient can be compensated. The manufacturer's value of temperature drift for the CL-2 is 0.1% per 1°. This is negligible for the average laboratory in temperate climates.

Another significant advantage is the maximum operating voltage of 300 V DC as compared to 90 V for most phototubes. The extremely small size of the crystal photocell is a distinct advantage in probe design. Since these cells are so inexpensive we believe that they will find increasing application in photometry.

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Improved resolution on paper chromatograms

In paper chromatography the original solution is commonly allowed to spread over a small circular area. This results, after development, in the spots corresponding to the different substances being irregular circles. Two substances with close R_F values cannot easily be distinguished since overlapping spots appear only as a rather more irregular circle. On one-dimensional chromatograms this is sometimes overcome by applying the original solution as a long streak, but this introduces difficulties and cannot be used for two-dimensional chromatograms. The method described here is applicable to one- or two-dimensional chromatograms and enables substances with very close R_F values to be distinguished.

The solution for analysis (10 μl) is applied to the paper and spreads as a small circular spot. This is dried and the operation repeated as many times as is needed to concentrate the material. The solvent (*e.g.* water if the original solution is aqueous) is applied in three 10 μl portions to the centre of the dried spot, drying between applications. As the solvent spreads out it carries the solutes to the periphery of the original spot, forming a ring with an empty centre. On running the chromatogram as usual, each substance runs at its characteristic rate forming a discrete spot which maintains its ring shape, though there is some spread and the ring changes to a solid circle at R_F values greater than about 0.4. It is very much easier to detect overlapping rings than overlapping circles, particularly if the centres nearly coincide, and one can be

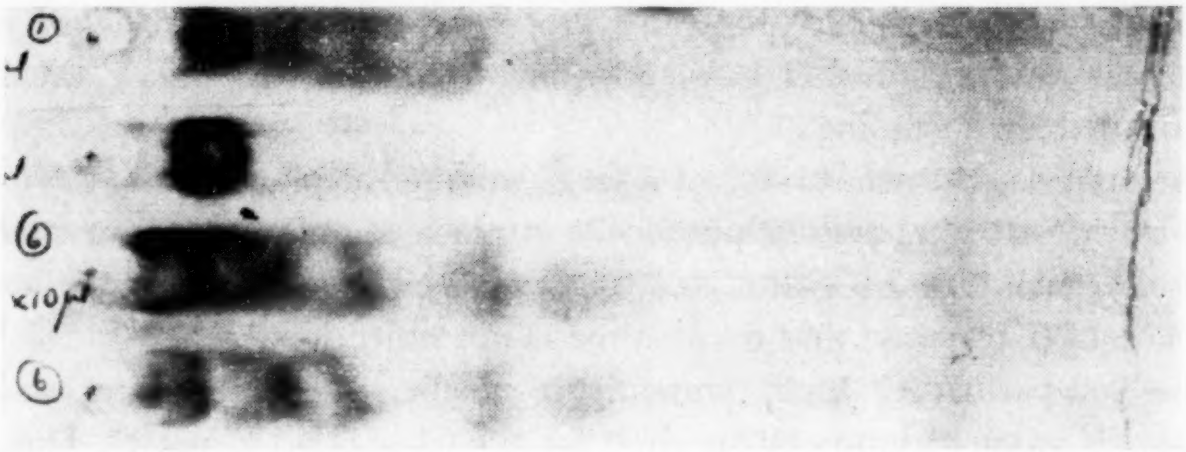

Fig. 1. One-dimensional chromatogram of phenolic substances in four specimens of urine, showing effect of ring formation. Solvent: isopropanol–ammonia–water (8:1:1). Untreated urine (30 μl) was applied on each cross. The solvent edge is marked.

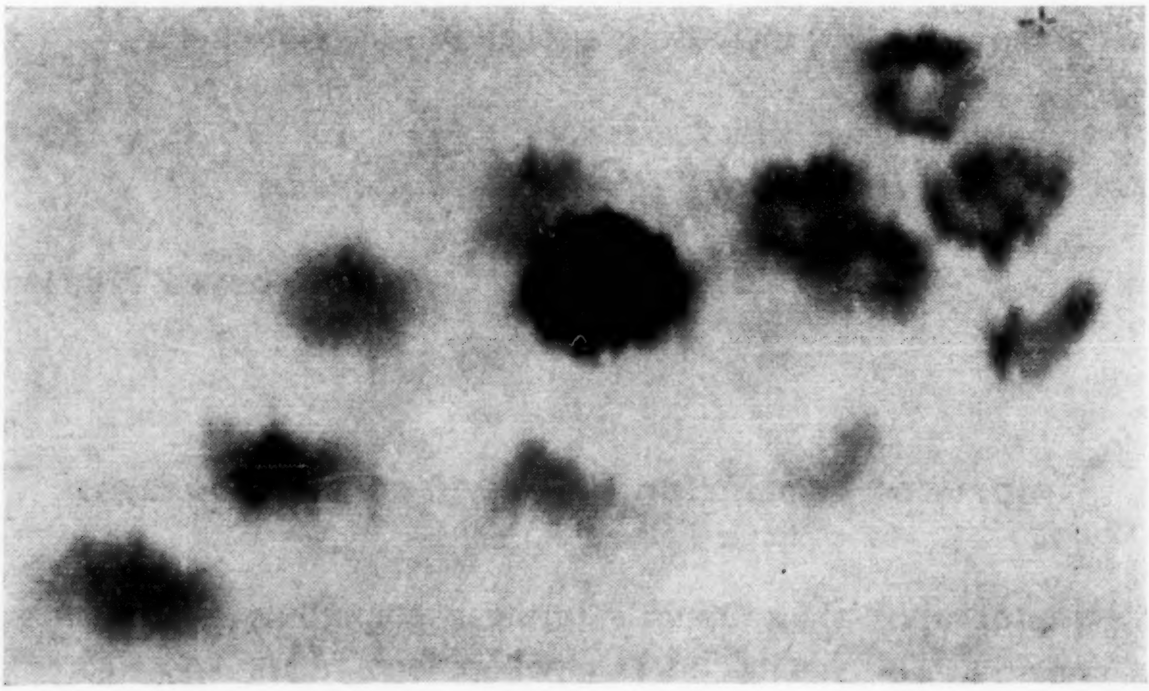

Fig. 2. Two-dimensional chromatogram of amino-acid mixture. Alanine is overloaded, normally it shows clear ring formation. This is just visible in the β-amino-isobutyric acid spot. Solvents: *p*-cresol–ammonia from right to left followed by collidine–lutidine from top to bottom. The amino-acid mixture was applied at the cross visible in the right-hand top corner.

correspondingly more confident of the identity or non-identity of two substances (Figs. 1 and 2). The amount of material per unit area is much higher in the periphery of a ring than in a circle of the same area, increasing the sensitivity of the method as well as the resolving power. Although the method fails for substances with high R_F values, it is in the low R_F region that one most often wishes to separate substances of similar R_F value.

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An improved technique for detecting spots on paper chromatograms with iodine vapor

The use of iodine vapor for detecting spots of various organic compounds on paper chromatograms has been a popular technique[1–5]. The disadvantage of this method lies in the fact that the paper background is also stained brown. For the examination of weakly stained spots as well as in the presence of tailing, the method was not found satisfactory.

On the assumption that the mechanisms through which the spots and the background are stained might be different from each other, attempts were made to reduce the staining of the background by treating the paper chromatogram with different reagents before being exposed to iodine vapor. It was found that filter paper impregnated with certain salts having acidic property, *e.g.* $(NH_4)_2SO_4$, $Al_2(SO_4)_3 \cdot 18H_2O$, McIlvaine's buffer of pH 2.2–4 (Na_2HPO_4 + citric acid), is far less stained than the untreated paper. Aluminum sulfate was found to be the most satisfactory; the impregnated paper strip remained virtually white even after being exposed to iodine vapor overnight. The spots, for example lipids like oleic acid, cholic acid or cholesterol, alkaloids including yohimbine or nicotine, and nitrogenous compounds such as *p*-anisidine and tryptophan, as well as the sulfur-containing compounds like methionine and glutathione, were stained as deeply as on untreated paper. The paper chromatogram treated in this way can be sprayed with starch solution whereby the iodine stained spots appear deep blue against a light blue background.

The procedure developed is as follows: The dried paper chromatogram is sprayed with a solution of aluminum sulfate in water (20 g $Al_2(SO_4)_3 \cdot 18H_2O$ in 100 ml water) on both sides until incipient dripping occurs. It is air dried and then hung overnight inside a jar containing some iodine crystals on the bottom. Exposure for 3 hours was found to be the shortest to obtain satisfactory results. The strip is again hung in air for 1 hour or longer to eliminate any superficially attached iodine and then sprayed with a 0.5% (w/v) starch solution. The background color becomes lighter as the strip dries. The color of the spots remains visible for over one month.

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Kombinationsmethode von Papierelektrophorese und Papierchromatographie zur Bestimmung von Aminosäuren*

Bei der Analyse von Aminosäuregemischen, in denen im Vergleich zu den neutralen Aminosäureanteilen sehr grosse Mengen saurer oder auch basischer Aminosäuren enthalten sind, ergibt sich bei der papierchromatographischen Auftrennung eine starke Überlappung der Komponenten mit benachbarten R_F-Werten. Die qualitative und quantitative Auswertung derartiger Chromatogramme ist dadurch ausserordentlich erschwert.

Zur Analyse solcher extrem zusammengesetzter Gemische entwickelten wir eine Kombination einer von uns modifizierten Papierelektrophorese mit unserer Keilstreifenpapierchromatographie[1].

Filtrierpapier Schleicher & Schüll 2045b, 40 cm lang und 4 cm breit, wird in Anlehnung an die Keilstreifenform bei der Papierchromatographie so zugeschnitten, dass ein Streifen entsteht, der über eine keilförmige Zunge zu einer schmalen Brücke führt, die sich dann wieder über eine Zunge auf die obere Breite erweitert, aber auf beiden Seiten der Brücke verschieden lang ist (Fig. 1). Die zu trennende Untersuchungssubstanz wird auf der kleinen Brücke aufgetragen; durch Schlitze an beiden Enden des Papierstreifens wird je ein kurzer Glasstab geschoben und der längere Teil des Streifens soweit zusammengerollt, dass die Brücke etwa in die Mitte der Elektrophoresekammer zu liegen kommt (Fig. 2). Der gesamte Streifen wird mit einem als Puffer dienenden Gemisch aus Pyridin–Eisessig–Wasser (30:100:5000) durch Besprühen gleichmässig befeuchtet; anschliessend werden die mit dem Glasstab beschwerten Enden in die mit dem oben verwendeten Puffergemisch gefüllten Elektrodengefässe gegeben. Es wird ein Gleichstrom von 150 V angelegt. Nach 3–5 Stunden wird der Streifen entnommen und getrocknet; der kürzere Teil des Streifens mit den darauf befindlichen basischen Aminosäuren (Fig. 3a) wird abgeschnitten und ein gleichlanger Papierstreifen an dieser Stelle angenäht (s. punktierte Linie in Fig. 1). Der gesamte Streifen wird erneut mit Puffer gesprüht und wieder in die Elektrophoresekammer gegeben. Anode und Kathode werden umgewechselt, sodass sich nach einer Laufzeit von etwa 19 Stunden die sauren Aminosäuren auf dem kürzeren Teil des Streifens befinden (Fig. 3b). Dieser Teil wird wieder abgeschnitten und zwar so, dass der verbleibende längere Teil die Form eines Langfilter-Keilstreifens erhält (Fig. 1, gestrichelte Linie). Die im Bereich der Brücke liegengebliebenen neutralen Aminosäuren werden auf diesem Streifen papierchromatographisch aufgetrennt.

Da die im Papier zurückbleibenden Reste des bei der Elektrophorese verwendeten Pyridinpuffers bei der papierchromatographischen Auftrennung eine Verschmierung der einzelnen Banden verursachen, müssen sie restlos aus dem Papier entfernt werden. Zu diesem Zweck werden die Streifen vor der Papierchromatographie in einem Vacuum-

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* Quedlinburger Beiträge zur Züchtungsforschung No. 40.

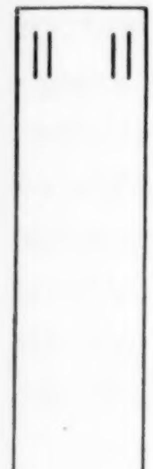

Fig. 1. Form des Papierstreifens zur Kombinationsmethode.

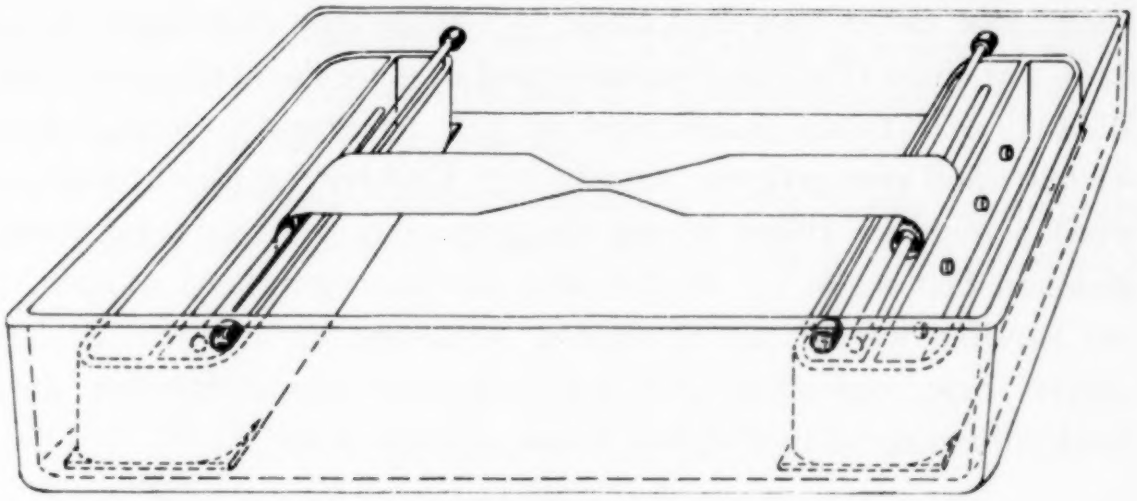

Fig. 2. Elektrophoresekammer.

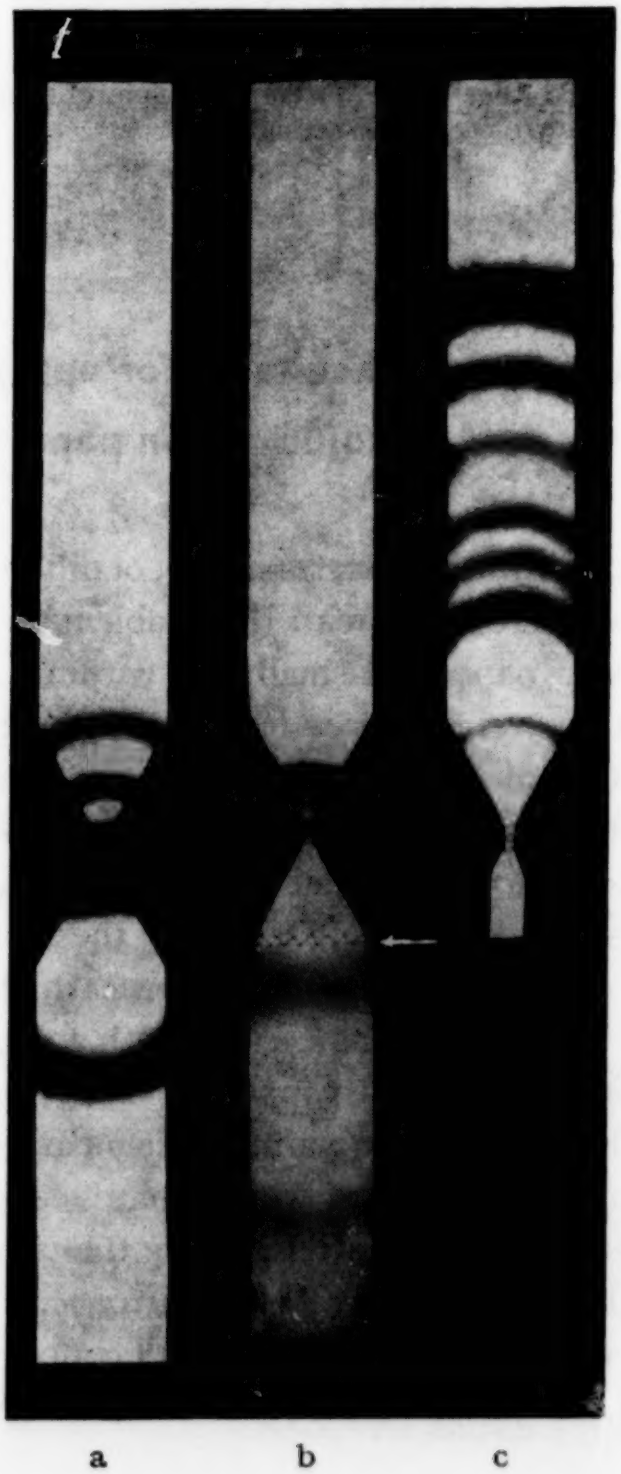

Fig. 3. (a) Papierelektrophoretische Auftrennung eines Aminosäure-Gemisches in saure, neutrale und basische Anteile. (b) Nach Umpolung saure Aminosäuren auf dem angenähten Streifen. (Nahtstelle = →). (c) Papierchromatographische Auftrennung der neutralen Aminosäuren.

Exsiccator unter öfterem Lüften ½ Stunde lang abgesaugt. 3–5 Keilstreifen werden dann auf einen Glasbügel gezogen und in einer flachen Schale* mit Butanol–Eisessig–Wasser (4:1:1) als Laufmittel in 2 × 17 Stunden papierchromatographisch entwickelt. Soll eine grössere Anzahl von Keilstreifen gleichzeitig entwickelt werden, so werden mehrere Bügel in ein Glasaquarium gehängt; auf diese Weise können zur gleichen Zeit bis zu 100 Keilstreifen chromatographiert werden. Nach dem Trocknen der Streifen werden die neutralen Aminosäuren auf dem Keilstreifen (Fig. 3c), die sauren bzw. basischen auf den abgeschnittenen Streifen durch Ansprühen mit Ninhydrinreagens in üblicher Weise nachgewiesen.

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* Die Glasgeräte zur Keilstreifen-Papierchromatographie werden hergestellt von VEB Glaswerke Ilmenau, Ilmenau/Thüringen.

An apparatus suitable for applying fairly large quantities of solutions on paper chromatograms

For the quantitative determination of dilute solutions of steroids by paper chromatography, we were faced with the problem of applying exact 0.5 ml quantities of such solutions on paper in a small spot within a reasonable period of time.

Of the several devices described in the literature, that of VAN GULIK[1] seemed to us the most promising. However, the time required to complete the operation under our conditions was found to be too long.

In order to circumvent this drawback, a modification of the VAN GULIK apparatus was constructed, enabling us to dry the solution during application by passing a centripetal current of warm air underneath the paper, around the spot of application (Fig. 1). The air-flow is measured with a rotameter A (for quantities of 10–100 l/min), then passes through a copper tube B, provided with a 220 V–500 W heating coil (from a hair-drier). The warm air then passes a thermometer C and is led to the copper "blow-cup" D, details of which are given in Fig. 2. The air passes through inlet E, the outer tube F and (after passing the paper) the inner tube G (screwed into F) and leaves the apparatus at H (see arrows). The space in the inner and outer tube is divided into four channels by small copper vanes in order to prevent whirling of the air. The various parts are mounted on a "philitex" table (25 × 35 cm).

The paper strip is firmly held down by a cover K. This cover is provided with a heavy metal ring, in the centre of which can be placed a capillary pipette, held in a vertical position by support L, and touching the paper.

The construction of the "blow-cup" (dimensions given in Fig. 2 in mm are quite essential) permits the passage of a strong air-current. For instance, at 60 l/min with air at 110°, a quantity of 0.5 ml of a steroid solution in 4-methyl-2-pentanone can be applied in a spot of 1 cm diameter, within a period of 2 min. Our pipettes are constructed to deliver such a volume in the given time when touching the paper. Of course, the amount of fluid, as well as the temperature and the velocity of the air-current may be varied to suit individual needs.

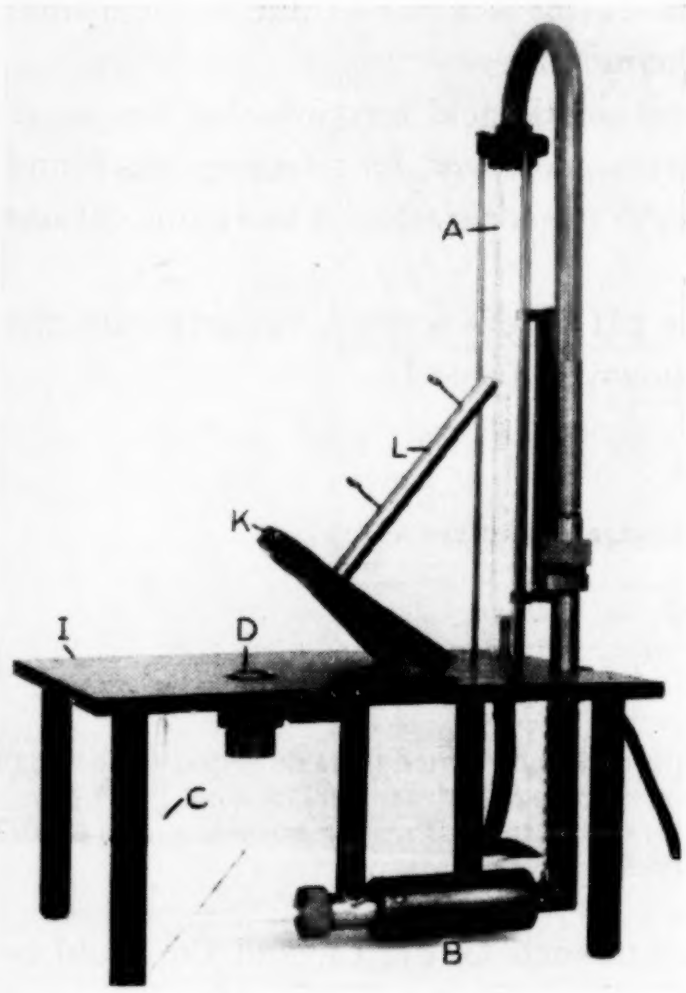

Fig. 1. Apparatus suitable for applying fairly large quantities of solutions on paper chromatograms.

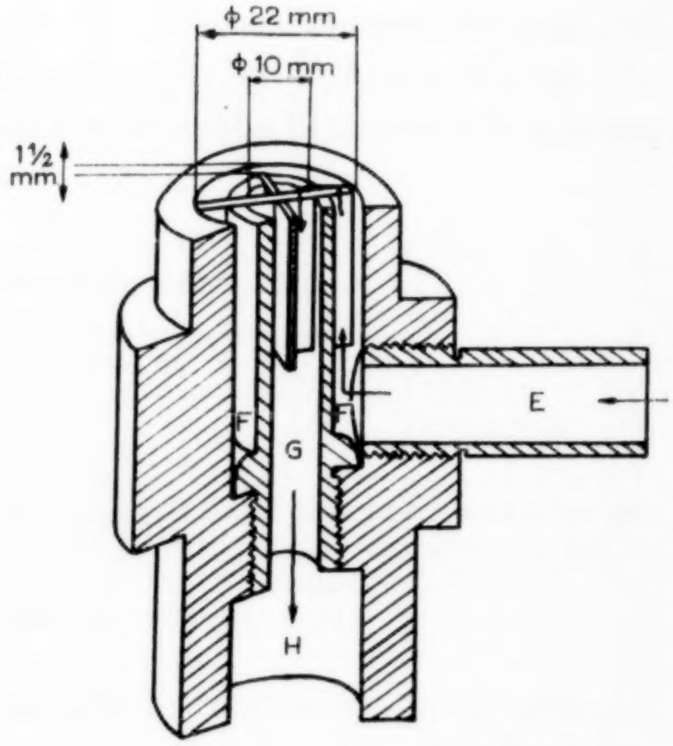

Fig. 2. "Blow-cup" of apparatus.

We wish to thank Mr. I. J. Stolk and Mr. J. de Vries for their skilful technical assistance in the construction of the apparatus.

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[1] W. J. van Gulik, *Nature*, 178 (1956) 994.

Received March 12th, 1959

Paper electrophoresis of oxine–metal complexes

MICHL[1] has shown that pyridine–acetic acid buffers not only have the advantage that they are volatile and that the increase in pH is constant, but also that separations of Cu^{++} and Cd^{++} are possible if this buffer is used; this is no doubt due to the formation of pyridine complexes of one or both of the metals.

POLLARD *et al.*[2] have used oxine in butanol–acetic acid mixtures for the paper chromatography of cations, notably of rare earths. However, no reference was found as to the possibilities of oxine–acetic acid buffers in the separation of inorganic cations by paper electrophoresis.

As with pyridine–acetic acid mixtures, the pH values increase linearly with the amount of acetic acid added to 1% oxine as shown in Table I.

TABLE I

pH VALUES OF MIXTURES OF OXINE AND ACETIC ACID

pH value	*% Acetic acid*	*% Oxine*
3.1	10	1
3.48	5	1
3.62	3	1
3.8	1	1

No interesting separations of transition elements such as Fe, Cu and Cd could be obtained as these ions precipitate in acetic acid–oxine while, when the pH is decreased by addition of HCl to about 2, they move as non-complexed cations.

The migration of the rare earths was found to be similar to that recorded in 1% citric acid[3]. La moves fastest, followed by the other rare earths approximately in the order of their atomic numbers. However, round spots without comets could only be obtained in the mixture 10% acetic acid–1% oxine. As in citric acid, there are fairly large differences between the mobilities of Nd and Sm, also between the mobilities of gadolinium earths and yttrium earths. In a moist-chamber apparatus (constructed by Jouan, Paris) with a paper length of 23 cm, separations of the following mixtures could be obtained: La–Y–Sc; La–Ce and Nd–Sm (see Fig. 1). The spots are readily revealed by drying the paper and then exposing it to an atmosphere of NH_3 which then yields fluorescent spots as described by POLLARD *et al.*[2]. However, the mixture acetic acid–oxine does not yield migration differences which are better than citric acid[3]; in the case of La–Ce–Pr the separation is even worse.

The variables of one of the successful separations, namely Nd–Sm, were studied in detail with a moist-chamber apparatus, 70 cm in length, using Whatman No. 31 extra thick paper. With 1000 V applied to the electrode vessels the paper burns through. With 750 V evaporation is excessive and the amount of liquid flow from the

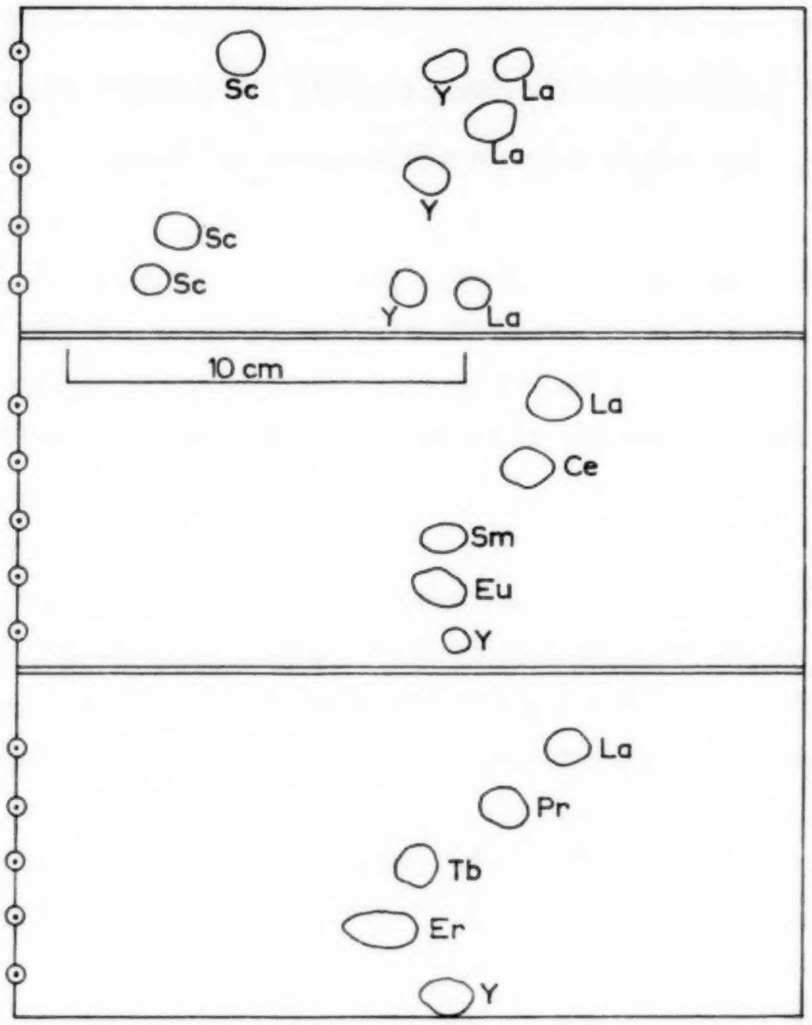

Fig. 1. The paper electropherograms with different pure rare earths and rare earth mixtures are run in the Jouan moist-chamber apparatus with 300 V for 4½ h with 10% acetic acid–1% oxine. Points of application are on the left. The movement is cationic. Paper: Arches 304. Three electropherograms, each with five spots are shown.

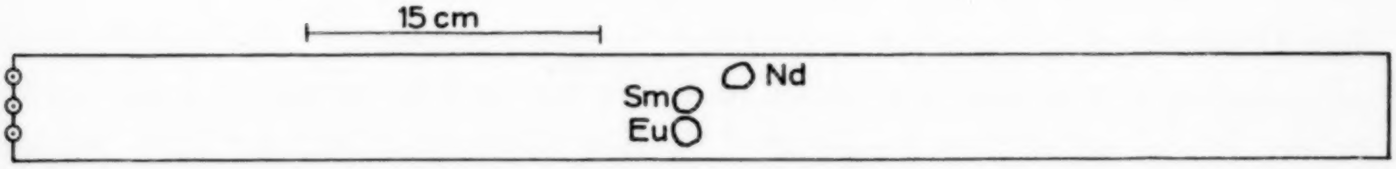

Fig. 2. Migration of spots of Nd, Sm and Eu placed side by side on Whatman No. 31 extra thick paper 70 cm long, and run with 300 V for 25 h.

electrode vessels is so large as to diminish the migration differences. The best separations are obtained with low voltages, 150–450 V and adequate times. Fig. 2 shows the migration of Nd, Sm and Eu with 300 V for 25 hours.

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[1] H. MICHL, *Monatsh. Chem.*, 82 (1951) 489.
[2] F. H. POLLARD, J. F. W. MCOMIE AND H. M. STEVENS, *J. Chem. Soc.*, (1954) 3435.
[3] M. LEDERER, *J. Chromatog.*, 1 (1958) 86.

Received February 25th, 1959

Production of anomalous compounds in paper electrophoresis by high concentrations of base*

We wish to report the appearance of an anomalous compound during the electrophoretic analysis of several reaction mixtures encountered in the synthesis of amino alcohols. This compound is attributed to the reaction of moderate concentrations of base with ninhydrin in the process of developing the electrophoretograms.

Method

A sample of the reaction mixture obtained in the reduction of glycylglycine with lithium aluminum hydride was placed on a 1.5 × 50 cm strip of S & S 597 filter paper previously saturated with potassium acid phthalate–sodium hydroxide buffer of pH 6.0 and of ionic strength 0.045. The electrophoretic separation was conducted at 600 V for 1 h on a Reco electrophoresis apparatus (model E-800-2). At the end of this time the paper strip was dried and the color developed with 0.25% ninhydrin in butanol saturated with water.

Results and discussion

The reaction mixture was shown to consist of at least four compounds giving a color with ninhydrin: unreacted glycylglycine, glycine, ethanolamine and an unknown compound which gave a yellow color with ninhydrin. The "yellow compound", as it will henceforth be called, had a negative charge at pH 6.0, migrating at approximately the same rate as ethanolamine, but in the opposite direction.

Identification of the components of the "yellow compound" was undertaken on a small quantity of this material which had been purified by means of a starch block separation. It was noted that the purified "yellow compound" did not have the same migration properties as it had in the mixture; that is, the purified "yellow compound" moved a short distance toward the cathode whereas the impure "yellow compound" moved rapidly toward the anode under identical electrophoretic conditions. An electrophoretic analysis of the acidic and basic hydrolysis mixtures of this compound indicated the presence of only one spot which occupied a position just slightly on the cathode side of the origin. No color area could be detected in the electrophoretic analysis of an unneutralized acid hydrolysis mixture.

The "yellow compound" was observed on the electrophoretograms of two other types of reaction mixtures dealing with the synthesis of N-glycylaminoethanol. In each case the pH of the mixture was on the alkaline side of neutrality.

A chance check of the effect of base on the electrophoretic migration of ethanolamine showed some rather interesting results. Not only was a spot visible for ethanolamine in its normal position, but there was also a color area in the position of that assigned to the "yellow compound". Further experimentation showed that sodium hydroxide was indeed responsible for the yellow color appearing on this electrophoretogram. In addition to sodium hydroxide, barium hydroxide, lithium hydroxide and potassium hydroxide also produced a spot corresponding in color and in position to that originally assigned to the "yellow compound". Lithium hydroxide was probably responsible for the production of this spot in the case of the reduction of glycylglycine with lithium aluminum hydride. The anomalous spot produced by these basic compounds was very similar to the "slow moving spot" described by WALDRON-EDWARD[1]. In this case the anomaly was caused by traces of sulfate.

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* Portion of a thesis presented by ELIZABETH C. SMITH as partial fulfillment of the requirements for the degree of Doctor of Philosophy. This report has been authorized for publication on October 9, 1958, as paper No. 2299 in the journal series of the Pennsylvania Agricultural Experiment Station.

Additional study indicated that the color formation was independent of the type of paper used. The exact concentration of base required for the production of the "yellow compound" is unknown. However, this spot appeared on electrophoretograms when 5–10 µl of 10% sodium hydroxide were placed on the paper strip.

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[1] DEIRDRE M. WALDRON-EDWARD, *Chem. & Ind. (London)*, (1954) 104.

Received February 13th, 1959

BOOK REVIEWS

Analytical Chemistry, Some New Techniques, by A. G. JONES. Butterworths Scientific Publications, London, and Academic Press Inc., New York, 1959, 268 pages, price 40 s.

The author states that he selected eight topics of special interest to him and presented them to non-specialists as introductions to these techniques without giving a complete survey. The eight topics are: Flame Photometry (47 pages), Differential Spectrophotometry (28 pages), Gas Chromatography (43 pages), The Use of Ion Exchangers in Analytical Chemistry (36 pages), Acid–Base Titrations in Non-aqueous Media (22 pages), Coulometric Titrations (20 pages), Differential Refractometry (18 pages) and The Determination of Oxygen and Hydrogen in Metals (28 pages).

The reviewer will confine himself to the chapters on gas chromatography and ion exchangers which are both excellent. The author demonstrates that these two topics can be fully explained and illustrated with numerous applications in as little as about 40 pages each, provided, of course, that the whole literature is not discussed. The chapter on gas chromatography contains adequate theoretical explanations and descriptions of apparatus employed as well as a number of well-chosen examples. Critical comments on various problems of gas chromatography are also made where they are needed, such as: "Many examples of the use of gas–liquid chromatography are now available in the literature but unless some way can be found at an early date of summarising all this information in a concise manner, much of it will be lost because the titles given to papers on this subject rarely betray the kind of separation found therein".

The chapter on ion exchangers again is a very good introduction to the subject. The reviewer feels, however, that the examples used to illustrate the various applications could have been better chosen. Much better separations of transition metals can be obtained on anion exchange resins than the separations of platinum metals quoted. The extensive work by K. A. KRAUS and coworkers should have been mentioned. It is too important to be overlooked.

Nevertheless the book can be strongly recommended to analysts or to advanced students. Only one misprint was noted: "Glueckauf" was written without a c.

M. LEDERER (Arcueil)

Gas-Chromatographie, von A. I. M. KEULEMANS, übersetzt und bearbeitet von E. CREMER. Verlag Chemie, Weinheim, 1959, 208 Seiten mit 26 Abbildungen. DM 24.—.

Die englische Originalauflage dieses Buches wurde schon in dieser Zeitschrift (Vol. 1, S. 203) von A. T. JAMES besprochen. Die Übersetzung und Bearbeitung von Frau Prof. CREMER ist ausserordentlich gut. Ergänzungen des jetzt schon zwei Jahre alten Originals sind an vielen Stellen angebracht sowie ein Anhang über Gas-Chromatographie an Adsorptionsschichten, dem Arbeitsgebiet der Übersetzerin.

Es ist zu begrüssen, dass in einer Zeit in der sogar eine Zeitschrift wie "Naturwissenschaften" manche Artikel in englischer Sprache veröffentlicht, eine Übersetzung manche sogenannte Anglizismen beibehält eher als neue Worte für schon wohlbekannte Begriffe zu formen, sodass Abkürzungen wie GLC und GSC und Worte wie Retentionsvolumen, Longitudinal Diffusion, Tailing und Tailing Reduktion dem Leser schon bekannt sind und sich keinerlei Schwierigkeiten bei dem Lesen der Originalliteratur ergeben, die momentan in diesem Gebiet hauptsächlich englisch ist.

Der Verlag Chemie hat die gewöhnliche praktische und gefällige Ausstattung diesem Band verliehen mit zahlreichen sehr klaren Illustrationen.

M. LEDERER (Arcueil)

Radioaktive Isotope in der Biochemie, by ENGELBERT BRODA with a preface by G. V. HEVESY. Verlag Franz Deuticke, Vienna, 1958, 326 pages, 30 illustrations and 13 tables.

The author, a radiochemist who has turned to biochemistry, has been able to write a book which contains the physical and radiochemical basis of radioisotope work as well as thorough discussions of applications of isotopes to biochemical problems.

The first eight chapters are devoted to the technical problems of radiochemistry and such topics as health hazards and the construction of counters etc. The material there presented should be read by every biochemist before employing radioisotopes. For instance the fact that overexposure for a short time can not be compensated by periods of work with inactive material is sometimes ignored even in radiochemical laboratories.

The following eight chapters are devoted to selected biochemical applications of radioisotopes. They contain a very extensive bibliography.

Difficult as it is to attempt a complete survey of radioisotope applications to biochemistry, it is remarkable how thoroughly the author has dealt with the topics selected and how well he has discussed them.

The book is well presented and printed and the reviewer did not notice any printing errors.

A. L. (Paris)

Announcement

Russian Journal of Inorganic Chemistry

Starting with the January 1959 issue, The Chemical Society is to publish, with the support of the Department of Scientific and Industrial Research, a cover-to-cover translation of the monthly journal, *Zhurnal Neorganicheskoĭ Khimii*, a publication of the Academy of Sciences of the U.S.S.R. The translation will be undertaken for the Society by Infosearch Ltd., and the Society has appointed Professor P. L. ROBINSON as Executive Editor of the publication. Professor ROBINSON will be assisted by an advisory panel of distinguished inorganic chemists.

The sale and distribution of the journal will be undertaken by Cleaver-Hume Press Ltd., 31 Wright's Lane, London, W.8, from whom a detailed prospectus giving the scope of this journal may be obtained.

Translations will be issued in monthly parts as soon as possible after the Russian original is available. The subscription rate will be £ 30 (U.S.A. $ 90) per annum, but Universities and Technical Colleges may subscribe at a discount of 25%. Single issues can be purchased at £ 4 (U.S.A. $ 12) per copy to all purchasers.

The Society also hopes to start the publication within the next year of translations of the Russian "*Journal of Physical Chemistry*" (*Zhurnal Fizicheskoĭ Khimii*) and "*Progress in Chemistry*" (*Uspekhi Khimii*).

NEW BOOKS

Gaschromatographie, by ERNST BAYER, Vol. X of *Anleitungen für die Laboratoriumspraxis*. (Springer Verlag, Berlin, 1959), iv + 163 pages, price D.M. 39.60.

Electrophoresis: Theory, Methods, and Applications, edited by MILAN BIER. (Academic Press, New York and London, 1959), xx + 563 pages, price $ 15.00.

Anorganische qualitative Mikroanalyse, by H. MALISSA AND A. A. BENEDETTI-PICHLER, Vol. I of *Monographien aus dem Gebiete der qualitativen Mikroanalyse*, edited by A. A. BENEDETTI-PICHLER. (Springer-Verlag, Vienna, 1958), 333 pages, price $ 11.65.

Protides of the Biological Fluids (Proceedings of the Fifth Colloquium, Bruges, 1957), edited by H. PEETERS. (Elsevier Publishing Co., Amsterdam, 1958), viii + 260 pages, price 45 s.

Protides of the Biological Fluids (Proceedings of the Sixth Colloquium, Bruges, 1958), edited by H. PEETERS. (Elsevier Publishing Co., Amsterdam, 1959), x + 330 pages, price 45 s.

Modern Trends in Documentation (Proceedings of a Symposium held at the University of Southern California, 1958), edited by MARTHA BOAZ. (Pergamon Press, London, 1959), 112 pages, price approx. 35 s.

Papierchromatographie in der Botanik, 2nd Ed., edited by H. F. LINSKENS. (Springer Verlag, Berlin, 1959), 428 pages, price D.M. 58.—.

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REVIEW

THE SEPARATION OF DIFFERENT TYPES OF HUMAN HAEMOGLOBIN

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I. INTRODUCTION

During the last decennium a large number of human haemoglobins have been described. Most of these haemoglobins are designated as abnormal, for they have not been found in the erythrocytes of the healthy normal human being.

Before 1949 two normal human haemoglobins were known: normal adult haemoglobin (Hb-A) and foetal haemoglobin (Hb-F). At birth, about 80% of the total haemoglobin of the infant consists of Hb-F. During the first months of life it is gradually replaced by Hb-A. In the blood of patients suffering from Cooley's anaemia, however, high percentages of Hb-F still remain during their whole life. In 1949, PAULING and coworkers[58] identified in the blood of patients suffering from the so-called "sickle-cell anaemia" a haemoglobin type differing from both Hb-A and Hb-F. The occurrence of this newly detected sickle-cell haemoglobin (Hb-S) appeared to be genetically determined. Since then, several other types of human haemoglobin have been described; these named in order of discovery are: Hb-C[34], D[35], E[37], G[14], H[65], I[69], J[81], K[9], L[1] and N[66]. A number of reviews have been written about the properties, distribution and clinical aspects of the human haemoglobins[59, 75, 5, 84, 39, 49, 50, 52, 57, 13, 29].

Apart from the clinical significance and the importance in anthropological studies, the occurrence of abnormal haemoglobins offers an interesting field of research to the biochemist. We are dealing here with a class of very closely related proteins and it is likely that many of these proteins occur as a result of a single mutation in one place in the system of genes responsible for the synthesis of normal human adult haemoglobin.

The family of human haemoglobins is extremely well suited for the investigation of the effect of a single mutation on protein structure. The main reason is that it is easy to obtain haemoglobin in a pure state, that is pure from the point of view of protein chemistry.

In order to study differences of protein structure between two closely related compounds it is essential that the different compounds should be identifiable.

Several methods have been used for the identification of haemoglobins. The first difference detected between Hb-A and Hb-F was the much slower denaturation-rate of the latter in alkaline solutions[44]. The so-called "alkali-denaturation method" was first used to estimate the amount of foetal haemoglobin in mixtures, and later to prepare solutions containing only foetal haemoglobin.

It was not until after the advent of electrophoresis as a general method that several other human haemoglobins were recognized, and some of them quantitatively estimated and isolated from mixtures in small amounts. Electrophoresis has since been the most generally used method for the identification and estimation of human haemoglobins. In some cases, when electrophoresis gives no satisfactory results, haemoglobins can be identified by solubility tests (differentiation between Hb-S and Hb-D).

The fact that the results obtained with nearly all the identification techniques strongly suggest that the erythrocytes of normal adults contain more than one type of haemoglobin, makes the matter more complicated. Different investigators have studied this apparent heterogeneity of normal human haemoglobin[4, 60, 20, 21, 47, 2, 24].

In this review we shall discuss some methods for the identification and estimation of human haemoglobins, and further we shall consider how far the different techniques are suitable for the isolation and purification of the different types.

Chromatographic techniques have proved to be very useful for the isolation, identification and purification of several proteins and polypeptides. Accordingly a large section of this review has been devoted to the chromatography of haemoglobins.

Techniques used for the further elucidation of the structure of a type of Hb that has already been detected, identified and isolated are not discussed here.

General properties and preparation of haemoglobin[83, 51]

The haemoglobin content of normal human adult blood is 14–16 g/100 ml. The pigment is accumulated in the erythrocytes, in which its concentration is about 32–35%. It is soluble in aqueous solutions with an ionic strength varying from 0 to very high values.

Haemoglobin is a conjugated protein with a mol. weight of about 67,000, consisting of a globin portion (96% by weight) and four "haem groups" (ferrous protoporphyrin complexes), which are readily split off in acid solution; the iron content of Hb is 0.34%.

Each haem group in the haemoglobin molecule can bind reversibly 1 molecule of O_2 or CO; the binding with the latter is by far the strongest. The absorption maxima in visible light are characteristic for the different haemoglobin derivatives; absorption maxima for non-oxygenated haemoglobin occur at 430 and 555 $m\mu$, for oxygenated haemoglobin (HbO_2) at 414, 541 and 576 $m\mu$ and for carbonmonoxy-haemoglobin (HbCO) at 418, 538 and 571 $m\mu$.

On addition of potassium ferricyanide oxyhaemoglobin is readily converted into ferri-(met-)haemoglobin, in which every haem group has gained an electron and can bind anions such as chloride or cyanide, but no oxygen or carbon monoxyde. Methaemoglobin cyanide is very stable and has characteristic absorption maxima at 414 and 542 $m\mu$.

Methaemoglobin formation also takes place in oxyhaemoglobin solutions on standing, especially at pH below 7, by auto-oxidation. This auto-oxidation can be avoided to a great extent by storage of the haemoglobin solutions in the CO-saturated state. HbCO should be used in almost all analytical or preparative work. HbO_2 is only used in the alkaline denaturation test or in some special solubility experiments.

Haemoglobin solutions are prepared as follows:

Fresh blood collected in solutions containing oxalate, citrate or heparin to prevent clotting, is centrifuged for 10 min at 600 *g*. The plasma is then pipetted off, the red cells are suspended in a 5-fold volume of 0.9% NaCl solution and the suspension is again centrifuged at 600 *g* for 10 min. This washing procedure is repeated 4 times and then the washed erythrocytes are lysed; after addition of 1 vol. of water and 0.5 vol. of toluene or octyl alcohol, the mixture is shaken vigorously for 1 min and allowed to stand overnight at 0–4° to complete haemolysis. After sharp and prolonged centrifugation (20 min, 3000 *g*) the stroma-protein (which amounts to 1% of the total protein of the erythrocyte and is the main impurity in haemoglobin solutions that have not been carefully prepared) is found between the upper (toluene) and lower layer. Toluene and stroma are pipetted off and the remaining haemoglobin solution is filtered through a thin layer of Celite 535. The resulting solution is clear and free of stroma as can be checked by adding to a sample an equal volume of saturated ammonium sulphate solution. No precipitate should appear.

Values for the haemoglobin concentration are obtained photometrically[12]; for an estimation a haemoglobin sample is first diluted and converted either into carbonmonoxy-haemoglobin (by bubbling CO gas through a solution diluted 200-fold with distilled water) or alkaline haematin (by diluting with 100 vol. of 0.1 *N* sodium hydroxide solution). The concentration of HbCO is estimated at 570 $m\mu$, that of alkaline haematin at 640 $m\mu$. The haemoglobin concentration of a solution prepared in this way described here, lies between 10 and 15%.

Carbonmonoxy-haemoglobin is prepared by bubbling carbon monoxide through the haemoglobin solution until a diluted aliquot no longer changes colour upon addition of $Na_2S_2O_4$. A drop of octyl alcohol is added to avoid foaming. The CO-saturated solution can be stored for some weeks at 0–4° or for much longer periods in the frozen state.

II. IDENTIFICATION AND ISOLATION TECHNIQUES

1. *Alkaline denaturation methods*[4, 18, 50, 42, 76]

The denaturation of oxyhaemoglobin by a large excess of alkali is a pseudo-monomolecular reaction. The reaction can be followed by determining (photometrically) the concentration of either the denaturation product (at 650 mμ) or non-denatured haemoglobin (at 576 mμ) at fixed times. On plotting log percentage of non-denatured haemoglobin against time the line obtained proves to be almost straight.

When a mixture of the haemoglobins a and b is denatured, the mixture obeys the equation:

$$(C_a + C_b)_t = (C_a)_0 \cdot e^{-k_a t} + (C_b)_0 \cdot e^{-k_b t}$$

If one velocity constant is large compared with the other, one term will become negligible within a few minutes and the semi-logarithmic plot becomes a straight line. Extrapolation of this line to zero time gives the percentual amount of the component with the lower denaturation velocity in the original mixture.

This method can only be applied for the estimation of foetal haemoglobin for this is the only alkali-resistant human haemoglobin known so far. The most precise estimation of low percentages of foetal haemoglobin in mixtures can be made by following the decrease of non-denatured haemoglobin at 576 mμ according to the modification of JONXIS AND VISSER[42]:

0.1 ml of oxyhaemoglobin solution or blood is diluted with 10 ml of water to which 2 drops of 10% ammonia are added. The extinction of this solution is measured at 576 mμ (E_b). 0.1 ml of the same haemoglobin solution or blood is diluted with 10 ml of 0.06 N sodium hydroxide solution, containing 2 drops of ammonia 10%. After homogenizing, the extinction at 576 mμ (E_t) is measured every minute during a period of about 15 min. After that the solution is placed in a water bath at 37° for 15 min, cooled again to room temperature and the extinction (E_e) is measured. The percentage of non-denatured haemoglobin at time t can be calculated with the equation:

$$\text{percentage non-denatured HbO}_2 = \frac{E_t - E_e}{E_b - E_e} \times 100$$

With this method Hb-F can be detected, when it is present in amounts above 1% of the total haemoglobin. When more than 5% is present it can be estimated with accuracy. JONXIS AND VISSER point out that the method is only suitable for laboratories with an accurate spectrophotometer, for the light absorption maximum of oxyhaemoglobin at 576 mμ is very sharp.

In SINGER'S precipitation technique[76], measurements can be carried out with a less accurate photometer:

One minute after the addition of 0.1 ml of an approximately 10% HbO_2 solution to 1.6 ml N/12 NaOH solution the denaturation reaction is stopped and simultaneously the denatured haemoglobin is salted out by the addition of 3.4 ml of a half-saturated ammonium sulphate solution containing 1 ml 10 N HCl/400 ml. The haemoglobin, remaining in solution is measured photometrically at 540 mμ.

The SINGER method gives values that are too high at very low percentages of Hb-F and too low at high percentages[41]. SINGER attributes too high "one-minute values" at low Hb-F concentrations to the presence of soluble degradation products of denatured Hb.

The precipitation method is of great advantage for preparative purposes[10, 72]. If a solution of alkali-resistant haemoglobin is contaminated with alkali-labile Hb, this alkali-labile fraction can be removed almost completely by making the solution alkaline for a few minutes:

To 2 vol. of a 10% HbO_2 solution 4½ vol. of 1/16 *N* KOH solution are added. The alkali-labile fraction is completely denatured and the alkali-resistant fraction only partly. After 2 min the solution is mixed with 16 vol. of a saturated ammonium sulphate solution, to which 0.95 ml HCl (sp. gr. 1.19) per 100 ml has been added. Denaturation ceases and the denatured Hb is salted out. The remaining haemoglobin consists to an extent of 100% of the alkali-resistant component.

Solutions of 100% Hb-F are prepared in this way from cord blood haemolysates[72].

The foetal haemoglobin, though not salted out, does not remain unaltered; it no longer crystallizes and is digested more rapidly by enzymes than native Hb-F.

It is clear that there are not many cases where the alkaline denaturation technique can be applied although it is the first method used to differentiate between two human haemoglobins and between haemoglobins of different species. Hb-F is the only human haemoglobin that can be detected, estimated and prepared by this method.

2. *Electrophoresis*

Moving-boundary method

As in other brances of protein chemistry, the so-called "moving-boundary method" of Tiselius has proved to be very advantageous in the field of haemoglobin research. The subject has been reviewed by experienced workers, such as ITANO *et al.*[39], so that only a brief summary of the most important features will be given here.

Good separations of different haemoglobin types can be obtained by application of the Tiselius method to 1% HbO_2 or HbCO solutions in cacodylate and phosphate buffers (pH 6.5, ionic strength 0.1) or in barbital buffer of pH 8.6 (ionic strength 0.06). In special cases buffers of very low ionic strength (0.01) are also useful.

Generally a potential gradient of 6–8 V/cm is applied for a period of 6 hours. Fig. 1, taken from ITANO *et al.*, gives a good illustration of the possibilities and the limitations of the moving-boundary technique in the analysis of haemoglobin mixtures. Good separations are obtained in many naturally occurring mixtures, such as Hb-A + S, A + D, A + E, F + E, F + S, A + C separate. On the other hand, Hb-S and C separate rather poorly, special buffer solutions[3, 71] are required to differentiate between Hb-A and F and Hb-S and D are not separable by the moving-boundary technique.

Some electrophoretical data of different haemoglobin types are listed in Table I.

TABLE I

ELECTROPHORETICAL CHARACTERISTICS OF DIFFERENT TYPES OF HUMAN CARBONMONOXY-HAEMOGLOBIN

	Hb type											
	H	*I*	*J*	*K*	*A*	*F*	*L*	*G*	*S*	*D*	*E*	*C*
Iso-electric point of HbCO in phosphate buffer, ionic strength 0.1	6				6.87	6.98		6.98	7.09	7.09	7.20	7.30
Mobility in phosphate buffer pH 6.5, ionic strength 0.1 ($\times 10^{-5}$ cm^2 V^{-1} sec^{-1})	— 1.5	1.7	2.0	= A	2.4	2.4	= A	≤ S	2.9	2.9	between A and S	3.2
Range of mobilities in barbital buffer, pH 8.6	H >	I >	J >	K >	A >	F >	L >	G >	S =	D >	E >	C

From the table it can be seen that some pH–mobility curves, for instance those of Hb-E and Hb-S cross each other. Therefore it is advisable to identify an abnormal haemoglobin type at not less than two pH-values.

In some cases, quantitative results are obtainable. As ITANO *et al.*[39] pointed out, percentages in unknown samples are best established by comparing with known artificial mixtures.

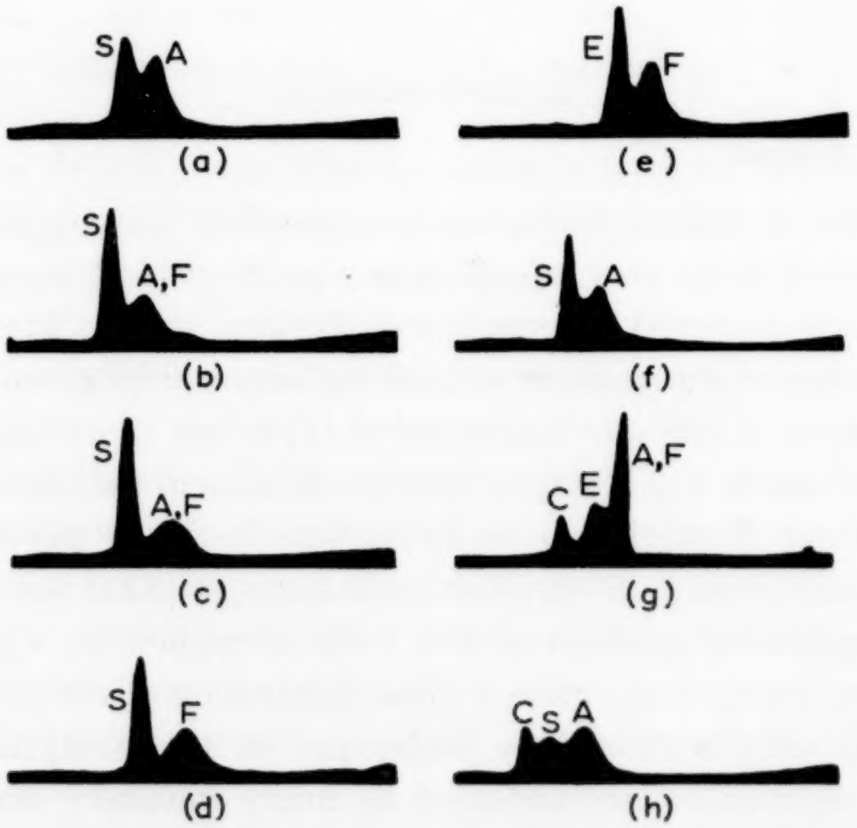


Fig. 1. Moving-boundary electrophoresis diagrams of human haemoglobin mixtures. Ascending boundaries; direction of migration from right to left; cacodylate buffer pH 6.5 and ionic strength 0.1. (ITANO *et al.*[39]). (a) sickle-cell trait; (b) sickle-cell anaemia, after transfusion of normal blood; (c) sickle-cell thalassaemia; (d) sickle-cell anaemia with about 40% foetal Hb; (e) Hb-E thalassaemia with about 40% foetal Hb; (f) artificial mixture of Hb-A and Hb-S, with about 40% Hb-A; (g) artificial mixture of Hb-C trait and Hb-E thalassaemia; (h) artificial mixture of Hb-C trait and sickle-cell anaemia.

Although the moving-boundary method is very important in mobility studies (especially at acid pH, as will be pointed out later) and in the determination of iso-electric points, it is not suitable for routine analysis. The (expensive) apparatus requires highly trained personnel and only a few analyses can be carried out in a day.

Zone electrophoresis

Zone electrophoresis, especially on paper strips or sheets, requires a much less complicated apparatus than free electrophoresis. Up to now, paper electrophoresis has been by far the most frequently applied routine method for the identification and semi-quantitative estimation of human haemoglobins. It has the advantage that on one sheet or strip of paper a number of haemoglobin samples can be run simultaneously; for one analysis, only a few tenths of a milligram of haemoglobin are required.

Paper electropherograms can be made in several ways: in one type of apparatus the sample runs on an "open" horizontal or vertical strip or sheet, from which evaporation is possible during electrophoresis[56]. In other types of apparatus, the so-called "pressure plate" or "closed plate" systems according to KUNKEL AND TISELIUS[46], a fairly thick paper sheet is pressed between two glass plates; in this way evaporation is prevented. We obtained results of excellent reproducibility[25,62] with the closed plate method according to SMITH AND CONLEY[77]. The procedure was as follows:

The apparatus consisted of a normal tank for horizontal paper electrophoresis, in which the paper sheet (Whatman No. 3 MM, 45 × 13 cm) is pressed between two siliconized glass plates (30 × 15 × 1 cm) by means of metal clamps. The buffer used is a barbital–sodium barbitalate solution pH 8.6, ionic strength 0.06 (composition: 10.3 g sodium barbitalate + 1.4 g barbital per l of distilled water). In the middle of the dry paper sheet a pencil line is drawn across the sheet. On this line (the starting line) a series of equidistant spots (the sites of application of the samples to be run) are marked. After the sheet has been soaked in the buffer solution it is blotted between two sheets of ordinary filter paper. Then the different haemoglobin samples are brought onto the paper (0.001 ml of a 10% HbCO solution for each spot), the sheet is clamped between the two glass plates, and the two ends of the sheet are hung in the buffer compartments of the electrophoresis tank. A potential of 350 V is applied to the platinum electrodes (the potential gradient in the paper is then approximately 10 V/cm). Good separations are generally obtained after a run of 8 h. In the apparatus described here, HbCO-A migrates in this time a distance of about 5 ½ to 6 cm towards the anode, sickle-cell haemoglobin (HbCO-S) migrates in the same time about 4 cm in the same direction. After completion of the run, the paper is dried in a hot air oven at 130° for 15 min and then stained in a solution of 0.4 g Amido Black in a mixture of 900 ml methanol and 100 ml acetic acid. After staining for 15 min, the paper is washed with a methanol–acetic acid mixture (also 9:1) until the background colour is faintly blue.

Under these conditions staining is not necessary for the identification of the haemoglobin sample, but it is very important for the conservation of the pattern. A semi-quantitative evaluation of the pattern can also be obtained with the stained sheet; the dye then is eluted from the spot in a few ml of a mixture of 50 ml of a 10% sodium carbonate solution and 50 ml of methanol.

Estimations by this method are, however, not very reliable. Much of the protein is adsorbed by the paper when the haemoglobin spot moves across it. This "trailing" caused by adsorption has always been the main disadvantage of paper electrophoresis.

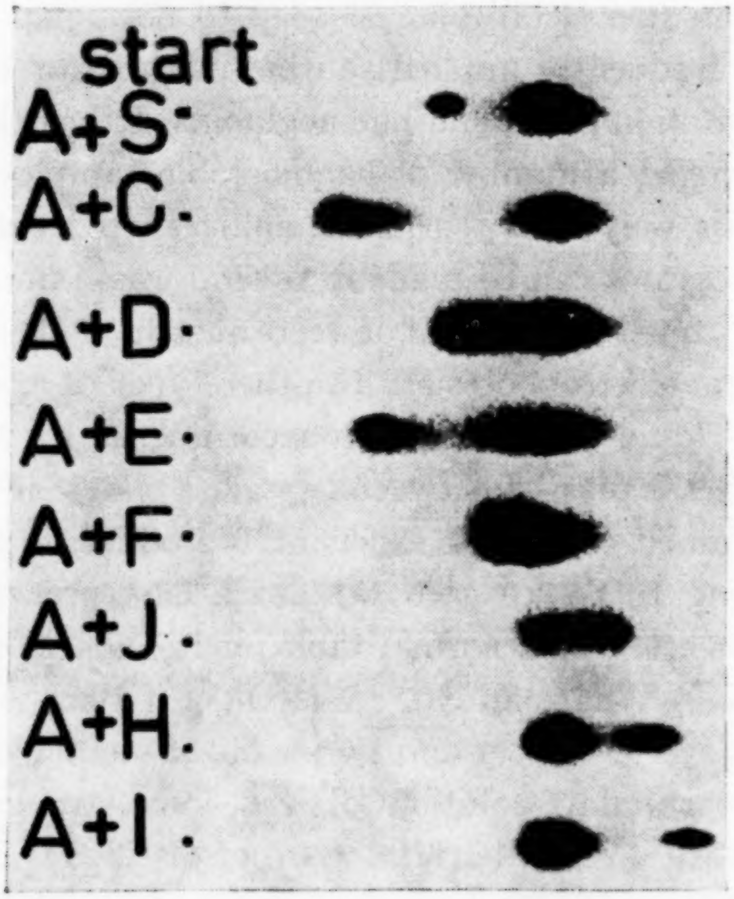


Fig. 2. A closed plate paper electrophoretic pattern of various mixtures of Hb-A with other human haemoglobin types. Whatman paper No. 3 MM; barbital buffer pH 8.6, ionic strength 0.06.

Therefore, we consider this method as very useful only for the qualitative analysis of haemoglobin mixtures. Separations obtainable with paper electrophoresis are illustrated in Fig. 2, in which patterns of mixtures of Hb-A with various other human haemoglobins are presented. Fig. 2. also gives a good impression of the practical utility of paper electrophoresis in the study of haemoglobinopathies.

The haemoglobins S, C, D, E, H, I, J can very easily be distinguished from Hb-A. On the other hand, resolution of mixtures of Hb-A with Hb-F or Hb-K, is rather difficult; Hb-D and Hb-S are indistinguishable at any pH and Hb-H and Hb-I only show a difference at acid pH. In order to obtain a good identification it is therefore necessary, as in the moving-boundary technique, to produce electrophoretic patterns at both acid and alkaline pH. However, good electrophoretic separations at acid pH can hardly be obtained because of the decreased stability of Hb at acid pH and the increased adsorption of Hb on paper.

Summing up the main features of paper electrophoresis in Hb determination, we can say that paper electrophoresis is a very simple, rapid and inexpensive routine method for the detection of most of the abnormal haemoglobins. However, not all haemoglobins are easily distinguished with this method and it is only suitable for working at alkaline pH.

Separations are disturbed by trailing, caused by adsorption; therefore in complex mixtures only the main components can be detected and quantitative results are difficult to obtain. Mobility studies are impossible because of the electro-osmotic flow, evaporation flow and the inhomogeneity of the electric field in the paper.

KOHN[45] introduced a *cellulose acetate strip* in zone electrophoresis, by which means adsorption was overcome to a great extent. His application of these cellulose acetate strips in electrophoresis of serum proteins and also of haemoglobins resulted in very clearly separated protein zones, not disturbed by trailing. We adopted KOHN's technique and found in preliminary experiments th[illegible] results were very well reproducible. A short account of our modification of KOHN[illegible] method is given here. The technique is an example of the "open strip" method.

The apparatus is shown in Fig. 3. The strips (5 × 10 cm) are stored in a cold barbital buffer solution, pH 8.6 (see p. 451), ionic strength 0.04. The same buffer is also used in the electrophoretic tank. Before use the strips are blotted with ordinary filter paper, placed in the tank and then a current of 2½ mA per strip is passed for half an hour before applying the sample. The haemoglobin samples can be applied in two ways: in spots (0.001 ml of a 10% HbCO soln./spot) or in lines (0.005 ml/line). The samples are applied 2½ cm from the anode end of the strip. After application of the samples, the same current as before is passed through for 3 h. Evaporation and electro-osmosis cause haemoglobin to migrate towards the cathode. The drying and staining procedures are the same as described on p. 451, except that the drying, staining and washing periods are all reduced three times. Before scanning, the blotted strips can be cleared by immersion in glycerol.

Results obtained in preliminary experiments with this technique are shown in Fig. 4. It can be seen, that the patterns show more detail than paper strip patterns; for instance in normal human adult haemoglobin the so-called A_2 fraction, which will be discussed later (p. 455), is clearly visible and can be measured to a certain extent. Scanning diagrams are easily obtained and do not indicate any trailing.

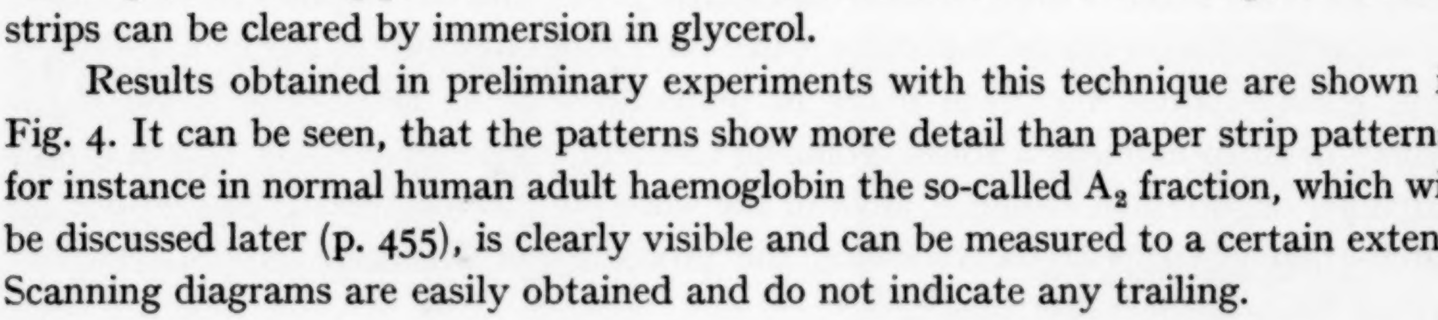


Fig. 3. Diagram of an electrophoretic tank, according to KOHN. Material: Lucite (Perspex). 1 = bridge; 2 = strip-holder; 3 = filter paper; 4 = strip; 5 = water. This apparatus is also suitable for strips of 5 × 20 cm.

References p. 484/486.

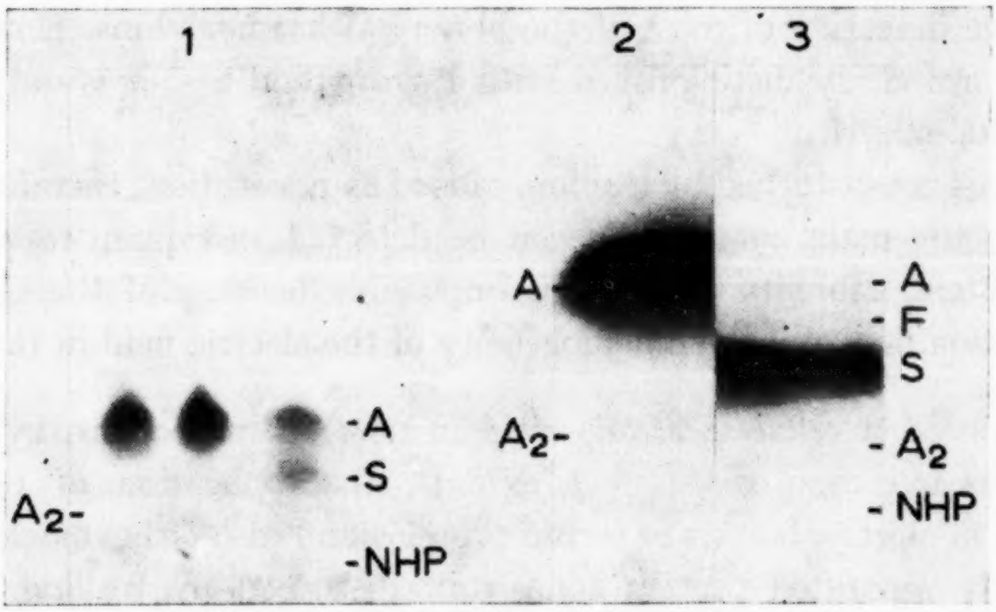


Fig. 4. Electrophoresis of various haemoglobin mixtures on cellulose acetate strips. Barbital buffer pH 8.6, ionic strength 0.04. 1. Spots of Hb-mixtures, run simultaneously on one strip. 2. Normal adult haemoglobin. 3. Haemoglobin in heterozygous sickle-cell-thalassaemia disease. N.H.P. = Non-haem protein.

The following disadvantages may be mentioned:

(1) The strips are very thin (thickness 13 μ) and therefore sensitive to evaporation.

(2) The strip material is brittle in the dry state, which makes it more difficult to handle than paper.

We expect that in spite of these disadvantages the method will prove to be very useful in work on haemoglobins.

Preparative zone electrophoresis

Many methods have been described for the separation of proteins on a preparative scale, use being made of differences in iso-electric points and electrophoretic mobilities. A comprehensive review on this subject has been given by Svensson[80] in 1949. Since then, great progress has been made in preparative zone electrophoresis in thick layers[48] or in columns of a zone-stabilizing medium, for instance agar, starch grain, starch gel or esterified cellulose. Most of the work on haemoglobins is carried out by starch-grain electrophoresis, following the procedure of Kunkel and Slater described below, or modifications of it[48]:

Potato starch is washed several times with barbital buffer solution pH 8.6, ionic strength 0.05, by repeated stirring, settling and decanting. Horizontal starch blocks are then prepared by pouring a very thick suspension of the starch into moulds of wax paper or plastic trays of various sizes. After the starch has settled, the excess buffer solution is removed with blotting paper and the surface is smoothed. A narrow slit is made across the block and the dialysed haemoglobin solution, either pure or mixed with starch is introduced. A glass or plastic plate is pressed on the surface of the block, and after equilibrating in a cold room, a current is passed through it over a long period. Generally, the starch block is connected with two large electrode vessels by thick blotting paper or cloth covered with cellophane or wax paper. After the run, the different fractions are cut out and eluted from the starch.

Good results are also obtained with a simple form of column electrophoresis[63]. In our laboratory we use starch grain columns of 40 cm length and 2 ½ cm diameter (Fig. 5). Columns are prepared by pouring a dilute suspension of the washed starch in barbital buffer (pH 8.6, ionic strength 0.03) into a cylindrical glass tube with a sintered glass disc at the lower end. The starch is allowed to settle and when most of the excess of buffer has flowed through the column, more of the starch suspension is introduced. This procedure is repeated until, after settling, the desired column height has been reached. During an equilibration time of a day, care should be taken that there is always some buffer solution above the column surface. Before starting an experiment, the excess of buffer is allowed to drain into the column; 2 ml of a dialysed 10% HbCO solution is carefully applied and washed as a zone of approximately 2 cm width to the middle of the column. Then the upper end of the column is connected with the anode vessel (Fig. 5) and a current of 10 mA is applied during 48 h. After completion of the electrophoretic run the column is placed above a fraction collector and the different zones are eluted from the column.

Starch grain electrophoresis is used for analytical as well as preparative purposes.

Very remarkable results have been obtained by KUNKEL AND WALLENIUS, who employed horizontal starch-slab electrophoresis[47] using barbital buffer, pH 8.6, ionic strength 0.05–0.1 (Fig. 6). The procedure permitted the electrophoretic separation of the main fraction of Hb-A from both a slower and a faster moving coloured component, regardless of whether HbO_2, HbCO or Hb was employed. It was possible to isolate these components by electrophoretic runs in which 15 ml of a dialysed 8% HbCO solution was applied in long slits. The slower component proved to be identical with the main fraction as regards visible and U.V. light absorption, as well as sedimentation constant. Both main and minor fractions, once purified, behaved as single

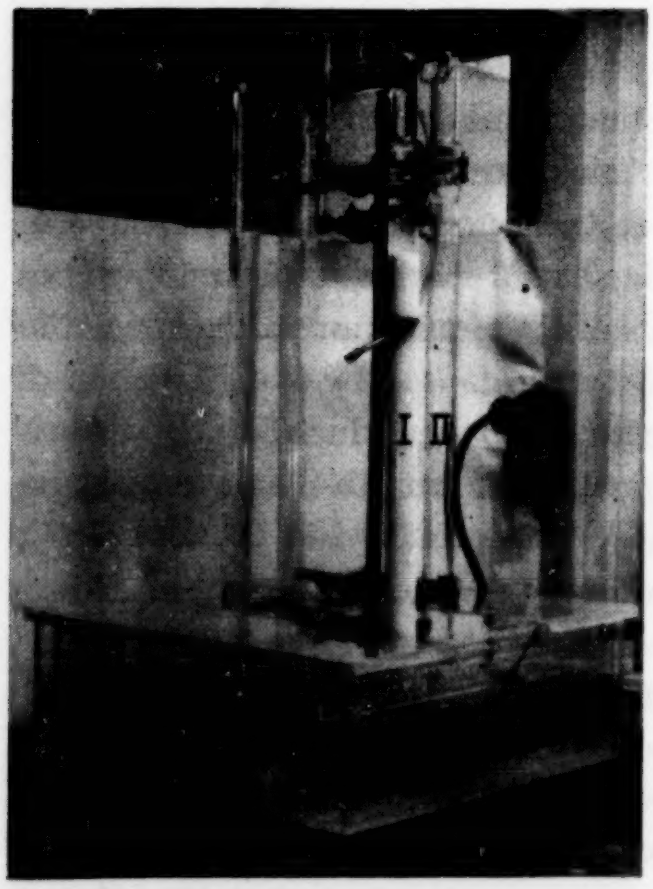


Fig. 5. Assembly for the column electrophoresis of haemoglobin samples. In one column (I) a haemoglobin sample is running; the other glass tube (II) is empty.

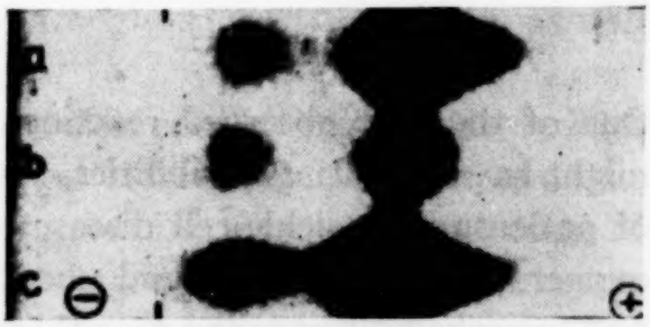


Fig. 6. Starch-grain electrophoresis of haemolysates of normal red cells (a and c) and E-trait cells (b). Barbital buffer pH 8.6; ionic strength 0.05. The lines indicate the site of application (KUNKEL AND WALLENIUS[47]).

components in repeated electrophoretic experiments. The slow fraction resembled Hb-E very closely in starch electrophoresis, and also in moving-boundary electrophoresis at pH's 8.6 and 6.5. Quantitative analyses of a number of blood samples of adults indicated that the mean value of the amount of the slower fraction was 2.6% of the total haemoglobin, with a range of 1.8 to 3.5%. Increased amounts were found in patients with thalassaemia minor.

The findings of KUNKEL AND WALLENIUS have been confirmed by various groups of investigators.

GERALD AND DIAMOND[16], investigating the blood of parents of patients with thalassaemia major, found an average value of 4.7% for the Hb-E-like fraction (designated A_2 fraction: $\sigma = \sqrt{\frac{\Sigma d^2}{n}} = 0.8$ %. In 200 normal healthy human adults MASRI, JOSEPHSON *et al.*[53] found an average of 2.55% Hb-A_2 ($\sigma = 0.62$%) and in patients with thalassaemia minor 6.49% ($\sigma = 1.5$%). Like KUNKEL AND WALLENIUS, they found a very low amount of Hb-A_2 in cord blood (0.88%, $\sigma = 0.15$%). As the various reported investigations indicate, starch-block electrophoresis can be very helpful in the diagnosis of thalassaemia minor, although great caution is advisable in drawing conclusions, for some other haematologic disorders are also apt to produce abnormal A_2 values[53]. A disadvantage of both the starch block and starch column methods is that they are laborious, and therefore not well suited for routine analysis. Perhaps in future the cellulose acetate strip will replace the starch slab for analytical purposes[45].

As a preparative method, zone electrophoresis has been found to be very advantageous for the isolation and characterisation of some haemoglobins and haemoglobin fractions, for instance for the preparation of "electrophoretically pure" Hb-A[64], Hb-H[23] and Hb-A_2[2], for amino acid composition analyses and terminal amino acid determination.

3. *Solubility*

One of the first observed reactions indicating that some abnormal haemoglobins might have deviating solubilities, is the so-called "sickle-cell reaction". Erythrocytes of patients with sickle-cell disease or "trait" lose their normal biconcave shape in oxygen-poor suspensions and change into sickle-shaped particles. After PAULING and coworkers[58] had found that these sickling cells contain an abnormal haemoglobin (Hb-S), it was believed that the sickling phenomenon is caused by the formation of highly ordered structures of the abnormal haemoglobin in the reduced state. Viscosimetric studies[2a] have revealed that in the deoxygenated form Hb-S can form large, birefringent aggregates, not only by itself but also with normal adult Hb and Hb-C. This tendency to aggregation is reflected in a markedly reduced solubility of deoxygenated Hb-S in various solutions.

The solubility of Hb-S and various other human haemoglobins were extensively studied by different investigators, employing the "variable solvent method" of ROCHE, DERRIEN and co-workers[67, 68].

In this method a constant volume of a Hb solution is added to each of a series of dilutions of 3.5 *M* phosphate solution, pH 6.5. After several hours of equilibration at 20°, the mixtures are filtered, and the extinction of the filtrates at 542 μ is measured and plotted against the phosphate concentrations of the phosphate–Hb mixtures.

Some examples of the solubility curves thus obtained are presented in Fig. 7 (taken from HUISMAN *et al.*[27, 73]). The curves of red cell haemolysates from blood of normal adults, from cord blood, and from blood of patients homozygous for the Hb-S and for the Hb-C gene are given. It is evident from the curves that there are significant differences between the various types of haemoglobin.

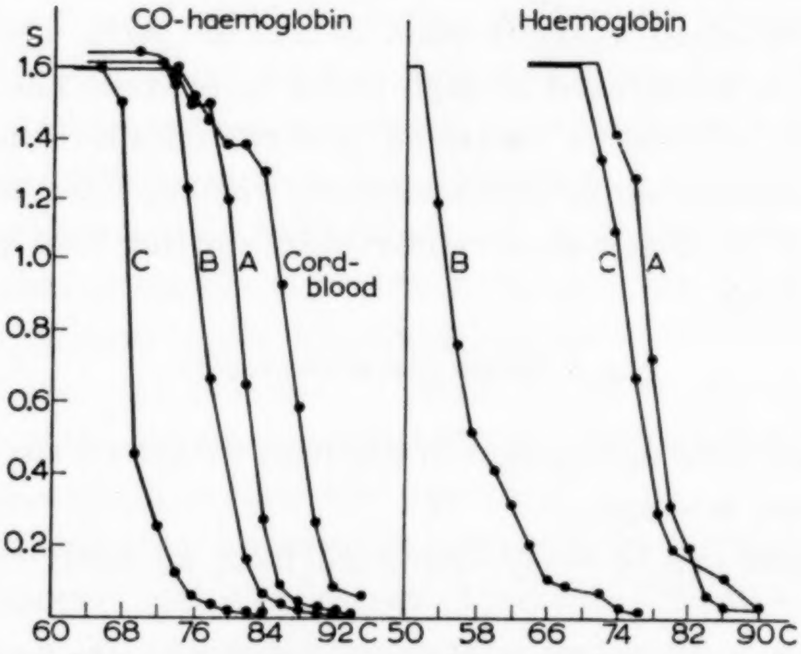


Fig. 7. Solubility curves of different human haemoglobins (HUISMAN *et al.*[27]). Abscissa: Percentage (by volume) of a 3.5 *M* potassium phosphate solution pH 6.5. Ordinate: Extinction at 543 mμ of the Hb solution after filtration.

The solubility curves of normal adult haemoglobin and cord blood haemoglobin obtained by ROCHE, DERRIEN and co-workers[68], strongly suggest that foetal as well as normal adult haemoglobin are not homogeneous.

ROCHE *et al.*[68] conclude from their experiments that cord blood contains, besides a low percentage of adult haemoglobin, different types of foetal haemoglobin, and that red cell haemolysates from normal human adults contain more than one type of haemoglobin, at least one of these components being of a foetal type. However, percentages of foetal haemoglobin, as large as measured by ROCHE *et al.*, have never been found in normal human adult blood by other techniques such as alkaline denaturation or an immunological method devised by CHERNOFF[10]. Partial alkaline denaturation of normal adult red cell haemolysate and estimation of the amino acid composition of the alkali-resistant part carried out by HUISMAN *et al.*[28] indicated that less than 1% of total Hb is Hb-F. Apart from differences of opinion about the explanation of solubility curves, the variable solvent method of ROCHE, DERRIEN *et al.* is a very helpful technique in the characterisation of any newly discovered haemoglobin type. For routine analyses it is unsuitable because it is too laborious. In many cases, the estimation of one or two points of a solubility curve, as in ITANO's simple modification[36], is already very helpful, for instance in differentiating between Hb-S (low solubility in the deoxygenated state) and Hb-D (solubility similar to that of normal adult Hb):

8 ml of a solution containing 281.2 g (1.62 mol.) K_2HPO_4 and 160.6 g (1.18 mol.) KH_2PO_4 per l, are placed in a 10 ml volumetric flask into which 100 mg of $Na_2S_2O_4$ have been weighed. After dissolution of the hydrosulphite, 1 ml of water is layered over this solution, then a solution of 50 mg HbO_2 is added, the flask is immersed in a 25° water bath, the volume brought to exactly 10 ml and finally the contents are mixed. The final phosphate concentration is 2.24 M. After appearance of a solid phase, the mixture is centrifuged at high speed or filtered. The amount of haemoglobin remaining in solution is measured photometrically. Under the conditions described, Hb-D remains completely in solution, whereas Hb-S is largely salted out. The solubility in 2.58 M phosphate is estimated by starting with 9.20 ml of phosphate solution instead of 8 ml.

4. *Column chromatography*

Chromatography has some advantages in common with zone electrophoresis:

(1) The technique is simple.

(2) The procedure can be easily converted from an analytical to a preparative scale.

(3) A series of analyses can be carried out simultaneously (more than one column can be placed above a time-operating fraction collector).

A combination of chromatography and zone electrophoresis has been used with great success in many biochemical investigations, for instance in the elucidation of insulin structure[70].

Protein chromatography differs in many respects from the chromatography of smaller molecules. The limited stability of proteins is important in this connection. In general, proteins are only stable in aqueous solution, in definite pH and salt concentration regions. Moreover, the denaturation rates increase steeply with rising temperature. These facts have the following consequences:

(a) Elution with organic solvents is in most cases impossible. One is restricted to the use of buffer solutions, the eluting properties of which can be controlled by varying the pH and salt concentration.

(b) Application of partition chromatography, apart from a few exceptions[79], is out of the question. In particular haemoglobin becomes denatured or decomposes readily in contact with organic solvents. Paper chromatography of haemoglobin with acetic acid–pyridine mixtures[4] results in haem being split off[63].

(c) Cooling during chromatography is advisable and sometimes necessary.

When the conditions necessary for obtaining a stable solution of a given protein have been fulfilled, this does not always mean that the protein can be chromatographed under these conditions without denaturation, for chromatography itself sometimes causes denaturation. During strong adsorption, great and irreversible changes may occur in the spatial structure of the protein molecules; elution then yields solutions of non-native, that is denatured, protein.

In general, weak adsorption is reversible. Furthermore, weak adsorption has the advantage that the eluted compounds are obtained in relatively high concentrations and that R_F values are high and therefore the time of operation short, which is important in work with material susceptible to thermal denaturation.

When selecting a suitable adsorbent for protein chromatography, ion-exchange adsorbents are apt to be preferred, for as far as elution fluids are concerned, only aqueous systems (buffer solutions) can be used.

Another requirement in protein adsorption is that the adsorbent should be of relatively small particle size. There are two reasons for this. The first is that protein molecules owing to their large dimensions cannot enter the framework of, for instance, synthetic ion-exchange resins, and therefore adsorption is restricted to the particle surface. To ensure good adsorption this surface has to be large. In the second place, because of the large dimensions of the protein molecules, the diffusion velocity from a certain place in the solution to the adsorbing surface is low. Therefore the path of diffusion must be as short as possible, in order to attain adsorption equilibrium rapidly. For this reason the liquid channels between the particles should be narrow and that, in turn, makes small particle size necessary.

Very good results have been obtained in haemoglobin chromatography by using carboxylic cation exchangers such as fine-grain Amberlite IRC-50 and carboxymethyl cellulose. These results will be discussed in the next sections.

Adsorption on Amberlite IRC-50

Amberlite IRC-50 (provided by the Rohm and Haas Company, Philadelphia), a copolymer of 95% methacrylic acid and 5% divinylbenzene, contains 9–10 mequiv. COOH/g dry weight. In work with proteins a fine mesh grade (XE-64 or 97) of this resin has been used extensively with great success (see the review by MOORE AND STEIN[54]). To understand the nature of the adsorption of a protein molecule on a weakly acid substance, such as Amberlite IRC-50, it is better to start, as BOARDMAN AND PARTRIDGE[8] did, by considering the pure ion-exchange adsorption of a small molecule such as a basic amino acid.

In this ion-exchange adsorption three equilibria are important:

$$\text{R—NH}_2 + \text{H}^+ \rightleftarrows \text{R—NH}_3^+ \qquad (1)$$

$$\text{—COONa} + \text{R—NH}_3^+ \rightleftarrows [\text{COO}^-]\,[\text{R—NH}_3^+] + \text{Na}^+ \qquad (2)$$

$$\text{—COONa} + \text{H}^+ \rightleftarrows \text{—COOH} + \text{Na}^+ \qquad (3)$$

Although a high percentage of the amino acid will be in the cationic form at low pH, the ion-exchange resin will be almost completely in the COOH form, so that no ion-exchange adsorption will occur. At a high pH value most of the resin is in the COONa form, but now the amino acid is no longer in the cationic state, so that again no adsorption takes place. Nearly neutral conditions will be optimal for ion-exchange adsorption, for then the resin can be in the sodium form to a considerable extent and the greater part of the basic amino acid will be in the cationic form.

Experiments carried out by BOARDMAN AND PARTRIDGE[8] on the adsorption of lysine (iso-electric point 9.7) on Amberlite IRC-50 were in good agreement with the theoretical considerations. Around pH 7 there was maximal adsorption of lysine from buffer solutions containing 0.34 g.ions Na+/l. Adsorption decreased gradually towards lower pH values and more abruptly at higher pH. Extending ion-exchange adsorption theory to poly-ions such as basic polypeptides and proteins one would expect qualitatively the same adsorption behaviour. Equation (2) becomes:

$$z\,\mathrm{Na}_S^+ + P_L^{+n} \rightleftarrows P_S^{+n} + z\,\mathrm{Na}_L^+ \qquad (2a)$$

in which z is the number of sodium ions exchanged by the adsorption of one poly-ion. If $[P_S]$ and $[P_L]$ are the activities of the poly-ions in the resin and the liquid phase respectively, $[\mathrm{Na}_S^+]$ and $[\mathrm{Na}_L^+]$ the activity of the sodium ions in resin and liquid phase, application of the law of mass action results in:

$$\frac{[P_S]\,[\mathrm{Na}_L^+]^z}{[P_L]\,[\mathrm{Na}_S^+]^z} = K \qquad (2b)$$

The adsorption coefficient C is proportional to $[P_S]/[P_L]$ $(= K[\mathrm{Na}_S^+]^z/[\mathrm{Na}_L^+]^z)$ and therefore as a rough approximation:

$$C = K_1 \times \left\{\frac{\mathrm{Na}^+ \text{ adsorbed by resin}}{[\mathrm{Na}_L^+]}\right\}^z \qquad (2c)$$

Equation (2c) indicates that the ion-exchange adsorption of poly-ions on a cation exchange resin is more sensitive to slight changes in cation concentration than that of monovalent ions. It is to be expected that the decrease of the adsorption coefficient with rising cation concentration will be steeper. The findings of BOARDMAN AND PARTRIDGE concerning the adsorption of cytochrome *c* (iso-electric point 10.1) at pH 7 were again in good agreement with their expectation[8].

However, when the adsorption was plotted as a function of pH it was found that at higher pH values the adsorption decreased steeply with increasing pH, while on lowering the pH it did not decrease but became very strong below pH 7, as can be seen from Fig. 8 (taken from BOARDMAN AND PARTRIDGE).

It was observed that the rapid rise of the adsorption coefficient below pH 7 coincided with a diminution of the amount of sodium ions taken up by the resin. Therefore it was believed that in this pH region adsorption is no longer caused by pure electrostatic forces, but chiefly by short-range forces such as those giving rise to hydrogen linkages, mainly between undissociated carboxyl groups of the resin and peptide bonds or other polar groups of the protein. The positive charge of the protein, which above pH 7 is probably the main cause of adsorption, rapidly becomes unimportant below that pH.

As for the haemoglobins (iso-electric points around pH 7 in 0.1 *M* phosphate buffer, and about 5 in 0.1 *M* citrate buffer), it can be expected that adsorption will always be due to secondary forces. In the pH region where these short-range forces become unimportant, there is no electrostatic attraction, because above pH 7 haemoglobins carry a negative net charge. Therefore, chromatography of haemoglobins must be carried out in a pH region somewhere between 5 and 7. Differences in adsorption coefficients will arise partly from differences in short-range forces (due to differences in the spatial structures) and partly from differences between electrostatic attraction or repulsion forces (due to differences in net charge at a certain pH).

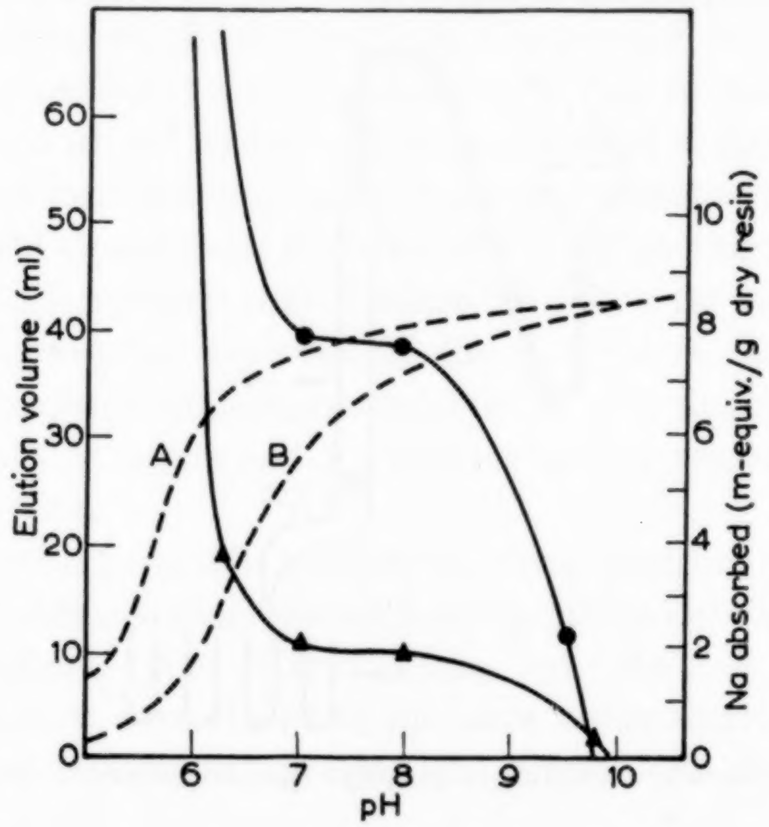


Fig. 8. Effect of pH on the elution of cytochrome *c* by sodium phosphate buffers. 6.0 mg samples were chromatographed on columns of IRC-50, 16.0 × 0.9 cm. Temperature 25°. Na^+ concentration: ● 0.25 g.ions/l; ▲ 0.34 g.ions/l. The broken lines show the amount of Na^+ taken up by the resin: A, from 1.0 *M* NaCl; B, from 0.1 *M* NaCl (BOARDMAN AND PARTRIDGE[8]).

Optimal conditions for a good separation in a given mixture have to be found empirically.

In haemoglobin chromatography on Amberlite IRC-50 the following procedure is generally followed.

Pre-treatment of the resin. The best way to make the commercial Amberlite IRC-50 XE-64 or 97 (passing a 150-mesh/in. sieve) ready for use has been described in detail by HIRS, MOORE AND STEIN[19]:

In order to remove the very fine particles, which would block the column, the following procedure is repeated several times: 1500 g of resin are stirred mechanically for 20 min with 3½ l of distilled water and then allowed to settle for 20 min after which the cloudy supernatant is decanted.

After this procedure the resin is stirred for 3 h in 3 l of acetone; fine air bubbles evolve and a yellow product is extracted. The ion-exchange resin is then cycled once or twice through the sodium form and finally stored, either in the acid form in aqueous suspension or in a concentrated buffer solution, or suspended in a NaOH solution. Regeneration of the resin is best carried out by heating for 6 h in 4 *N* HCl, washing with water, heating for 6 h in 4 *N* NaOH solution and again washing with water.

Before use, the purified resin can be roughly equilibrated with the buffer used in the experiment, by repeatedly suspending it in a large excess of buffer. Alternatively, the acid form of the resin can be used without pre-equilibration; saturation with the buffer is then performed on the column.

Preparation of the columns. The columns are prepared at the temperature at which the chromatographic experiment will be carried out. A suspension of the resin in 2 to 3 volumes of buffer solution or distilled water is poured, in small portions at a time, into a vertical glass tube fitted with a sintered glass disk at the lower end. After the first portion of the resin has settled, more of the slurry is poured into the tube, until after settling the desired column height has been reached. A separatory funnel containing the developing buffer saturated with carbon monoxide is connected to the tube (Fig. 9) and buffer is passed through the column at a high flow rate until the pH of the effluent is equal to that of the buffer entering the column. Then, by lowering the separatory funnel, the flow rate is adjusted to the value that will be used in the experiment (½–¾ ml/h per cm^2) and maintained for several hours, a check also being kept on the pH during that time. Columns prepared in this way, can be stored for some weeks, but only in the cold and if care is taken that there is always some buffer solution above the resin surface.

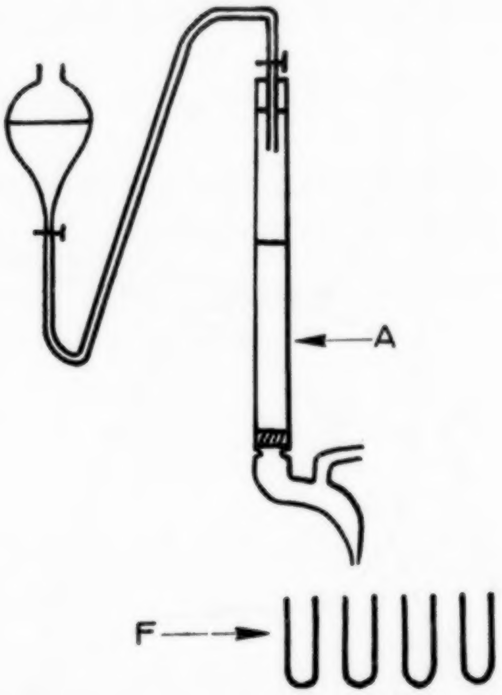


Fig. 9. Assembly for elution chromatography. A = adsorbent. F = fraction collector.

Chromatographic procedure. At the beginning of a chromatographic experiment the buffer solution standing above the resin surface is allowed to drain into the column. As soon as the surface is "dry", the sample to be chromatographed is added very carefully, drop by drop, to avoid disturbance of the resin surface. The sample consists of a 1–4% solution of HbCO in water, the volume employed for a 1 × 10 cm column being ½–1 ml. The HbCO solution is allowed to drain into the column by gravity, then about 1 ml of developing buffer is dropped carefully on the resin surface, after which a small plug of cotton wool is placed on the top of the column (to protect the resin surface from being disturbed by the next manipulations); then the glass tube is rapidly filled with buffer and finally connected with the separatory funnel.

The haemoglobin concentration in the effluent is estimated by diluting the ¼ or ½ ml fractions to 4 ml with distilled water and measuring the optical density at 570 mμ, or, for low concentrations, at 418 mμ (Soret line) in a spectrophotometer.

All results reported by different investigators have been obtained with this, or a very similar, procedure.

The results obtained by BOARDMAN AND PARTRIDGE[8] with mixtures of sheep foetal HbCO and bovine HbCO are very instructive. Fig. 10 shows elution curves of experiments performed at 25° and 2°. In both diagrams a third peak can be seen besides the two peaks corresponding to the two components of the mixture (peak A being sheep foetal HbCO and peak B bovine HbCO). This third peak was obtained by eluting the brownish material that was left behind on the column by both components. From the absorption spectrum of the material in the third peak it was concluded that it consisted of methaemoglobin. It was found that formation of methaemoglobin could be suppressed by working at low temperature with freshly prepared HbCO solutions.

In experiments with human haemoglobins, using mixtures of human adult and foetal HbCO, we[30,62] obtained diagrams such as that shown in Fig. 11. It can be seen that the two haemoglobin types separated very well; Hb-A is, however, eluted in much lower concentration than Hb-F. In the same buffer solution good separations could also be obtained of mixtures of HbCO-A and HbCO-S (sickle-cell Hb). Hb-S

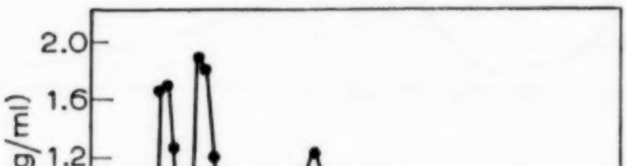


Fig. 10. Chromatograms of a mixture of sheep foetal HbCO and bovine HbCO on 0.9 × 5.0 cm Amberlite IRC-50 columns. Buffer: sodium citrate pH 5.81, Na$^+$ conc. 0.34 g.ions/l; after 1.2 ml changed to sodium citrate pH 6.5, Na$^+$ conc. 3.2 g.ions/l. (a) Temperature 25°; (b) Temperature 2° (BOARDMAN AND PARTRIDGE[8]).

proved to be more strongly adsorbed than Hb-A; it could be eluted in a concentration comparable with that of Hb-A when a pH 6.5 citrate buffer, containing 0.25 g.ion Na^+/l, was used. The fourth haemoglobin type investigated was Hb-C, which showed the strongest adsorption that has been observed until now; it could be eluted with a pH 6.5 citrate buffer, 0.30 g.ion Na^+/l. The yields of HbCO could be improved from about 85% at 10° to almost 100% at 0°. We used the chromatographic procedure to isolate Hb-F from the blood of a patient suffering from Cooley's anaemia, in order to estimate the amino-acid composition of this haemoglobin[31].

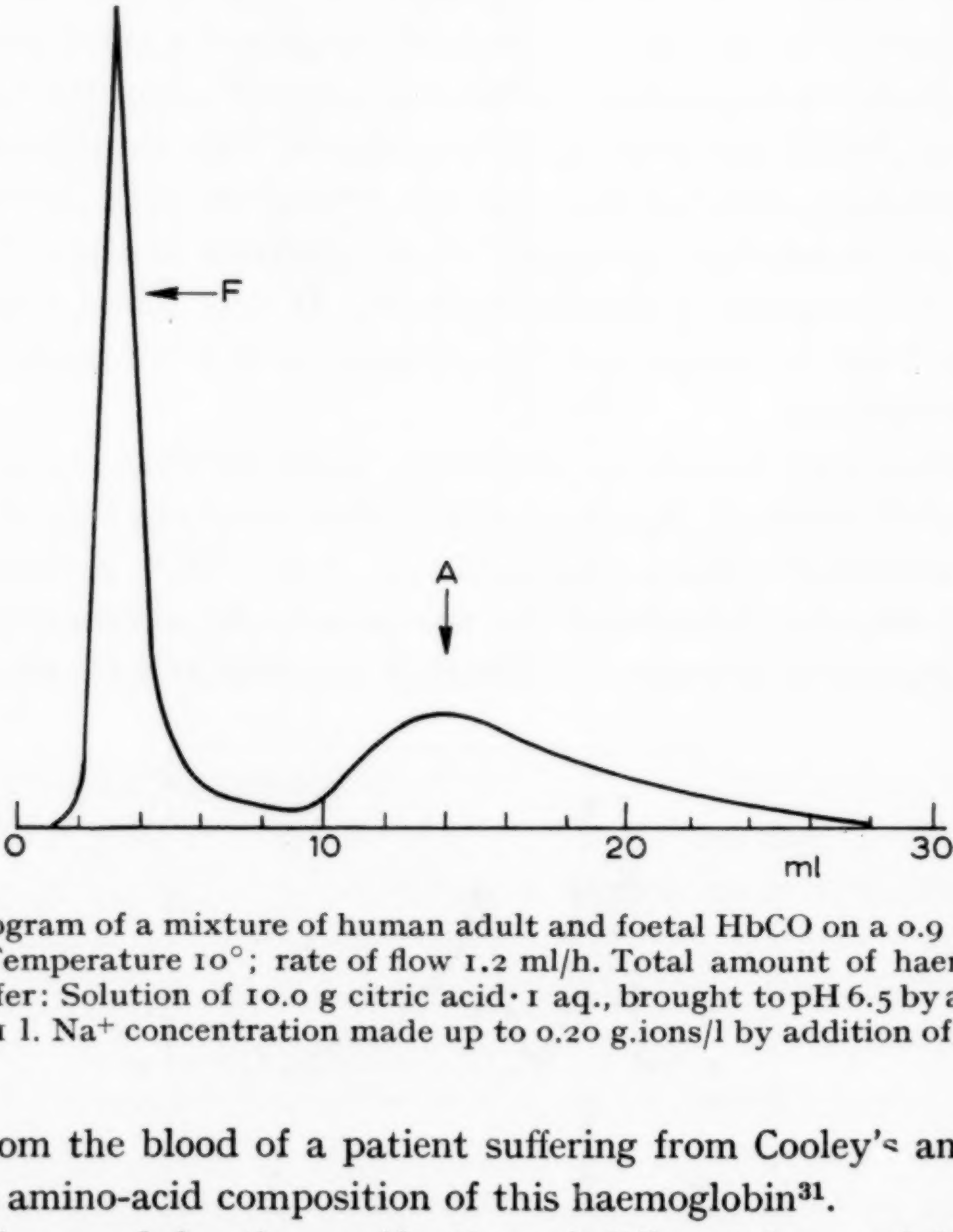


Fig. 11. Chromatogram of a mixture of human adult and foetal HbCO on a 0.9 × 7 cm Amberlite IRC-50 column. Temperature 10°; rate of flow 1.2 ml/h. Total amount of haemoglobin approximately 5 mg. Buffer: Solution of 10.0 g citric acid·1 aq., brought to pH 6.5 by addition of NaOH, diluted to 1 l. Na^+ concentration made up to 0.20 g.ions/l by addition of solid NaCl.

Besides being used for the purification of different haemoglobin types before further characterisation, chromatographic procedures may be of importance in the identification of abnormal haemoglobins in clinical serial analyses. The procedure described in the foregoing part is, however, still too complicated and time-consuming to be suitable for routine analysis. Therefore we simplified the method in the following way[25, 30, 62]:

The cylindrical glass tubes are replaced by flat Lucite (Perspex) cuvettes (20 × 3 × 0.5 cm inside dimensions) placed in a rack (Fig. 12) in which they can easily be moved upwards or downwards. The cuvettes are connected with the buffer vessel by means of siphons.

The chromatographic procedure is as follows:

Amberlite columns of 15 cm height are prepared as described above (p. 462). The upper few mm of resin are equilibrated with 25 ml of the developing buffer (a 20-fold dilution of a sodium citrate–citric acid solution, pH 6, containing 1 mole of trisodium citrate per l). The resin is further equilibrated with the buffer during the development of the chromatogram. As a result of the strong buffering properties of the resin (it contains about 10 mequiv. H^+ ions per g dry weight, that is about 2 mequiv. H^+ per cm column height), a pH and Na^+ concentration gradient develop on the column during chromatography. At first this gradient is very steep, but it becomes flatter with time. After passage of 250 ml of the developing buffer, however, the pH difference between the top and the bottom of the cuvette is still 0.5 pH unit[63].

After 25 ml of buffer have passed through the column, 2–4 ml of an aqueous solution of 10–15 mg HbCO are carefully applied on the surface of the resin and allowed to drain into the column; then about 5 ml of citrate buffer are carefully applied. When there is still a layer of buffer solution above the resin surface, this surface is protected by placing a piece of cotton wool on it. Finally the cuvette is connected with the buffer vessel by means of a siphon and the rate of flow is adjusted to about 10–15 ml/h. It is advisable to place the whole assembly in a cold room or refrigerator at 0–4°, to avoid methaemoglobin formation (see Fig. 14).

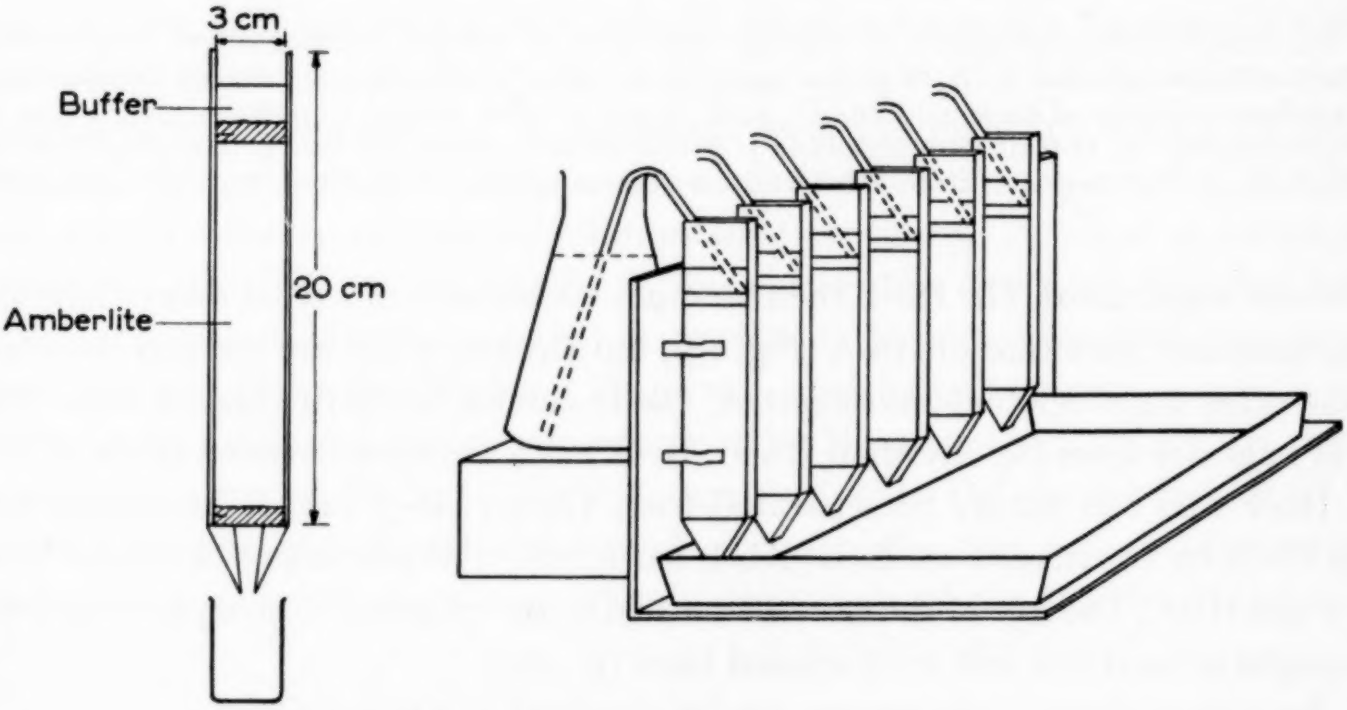


Fig. 12. Technical equipment for chromatography in flat cuvettes.

When 200 ml of buffer solution have passed through, a good separation of an artificial mixture of Hb-A and Hb-F or Hb-A and Hb-S is in general obtained: two horizontal well separated zones become visible. In Fig. 13, separations carried out with some artificial and naturally occurring mixtures are shown. It appeared that when cuvettes were used under the conditions described above, very good separations of haemoglobins F, A, S and C could be obtained; the relative migration velocities proved to be fairly constant. If the rate of displacement for Hb-A is taken as 1.0, the rate for Hb-F is 1.3; for Hb-S 0.7; for Hb-C 0.3.

The separation of Hb-E from Hb-A is attended by more difficulties; a good separation could only be realized when a low rate of flow (5 ml/h) of the developing

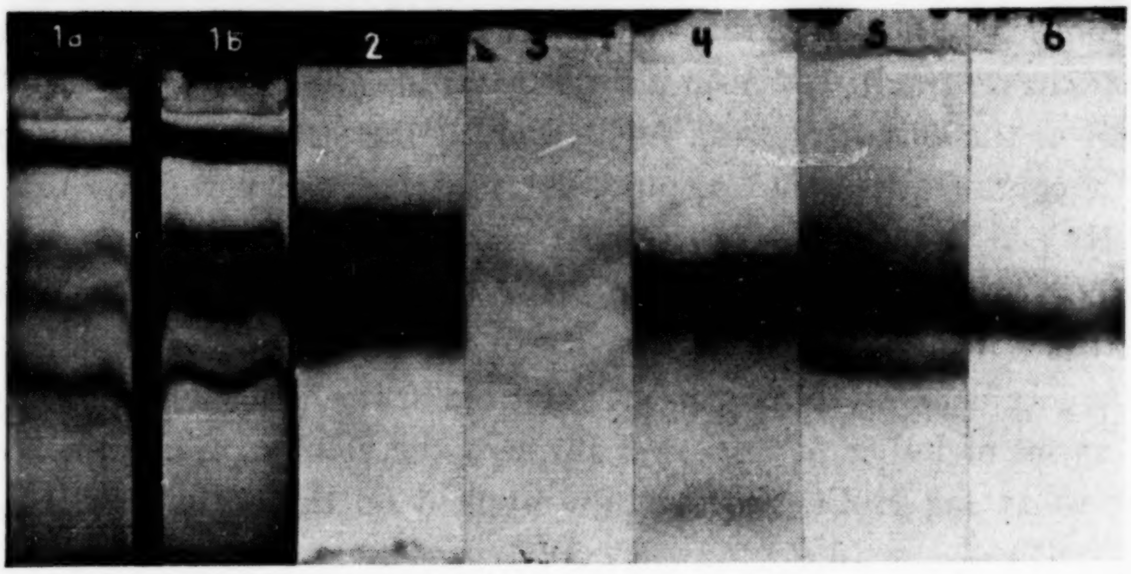


Fig. 13. Cuvette chromatograms of various mixtures of human haemoglobins. 1a: Artificial mixture of haemoglobins F, A, S and C (named in order of decreasing rate of displacement). 1b: Artificial mixture of haemoglobins F, A, E, S and C (also named in order of decreasing rate of displacement). 2: D-trait haemoglobin. 3: Hb-E thalassaemia disease. 4: Haemoglobin from Hb-H trait. 5: Haemoglobin from Hb-I trait. 6: Haemoglobin from Hb-J trait (no separation).

buffer was maintained. The Hb-E then becomes visible as a somewhat slower migrating zone, compared with that of Hb-A (Fig. 13), but the two zones are scarcely separated. Of the other haemoglobins investigated, Hb-D cannot be distinguished from Hb-S, Hb-H migrates more rapidly than Hb-F, Hb-I has a velocity between those of Hb-A and Hb-F and can hardly be separated from Hb-A; Hb-J cannot be distinguished from Hb-A by the cuvette method; Hb-L has a rate of displacement between those of Hb-S and Hb-C. The role of the cuvette method in the qualitative analysis of unknown haemoglobin mixtures will be discussed later (p. 481).

Semi-quantitative information can be obtained in various ways:

(1) By determining the optical density by scanning diapositives of the cuvettes, as in the direct scanning of paper electrophoresis strips[17]. Some scanning diagrams are given in Fig. 14. It can be seen that the results are far better at 0° than at 20°. Methaemoglobin formation in particular proves to be largely suppressed at low temperatures.

(2) By means of a more rapid procedure in which cuvettes with a removable front plate are employed. After development of the chromatogram, the front plate of the cuvette is detached, the separated zones are cut out and eluted from the resin into separate small chromatographic glass tubes with a more concentrated citrate buffer (a 3-fold dilution of the stock solution described on p. 465). The concentration of haemoglobin in the eluates is estimated photometrically. The method is of course also suitable for small scale preparative work.

By both estimation methods, relative amounts of haemoglobins in mixtures as low as 10% can be measured with reasonable accuracy. Zones containing ½ mg Hb (in general constituting 5% of the total amount of Hb) can be detected by visual inspection but cannot be easily measured.

In many cases, when the flow rate of the developing buffer is low, a second zone besides the main fraction becomes visible in a chromatogram of normal adult Hb and also of cord blood Hb; this second zone migrates with a somewhat higher velocity. In Fig. 15, the secondary fractions of Hb-A and Hb-F (designated as Hb-a and Hb-f respectively) can be seen. The occurrence of these zones can lead to wrong interpretations of chromatograms; Hb-a can be mistaken in this way for Hb-F. Any uncertainty can, however, always be resolved by adding a small amount of cord blood haemoglobin to the mixture in a second run, because this gives rise to a second small zone in front of the zone originally taken for foetal Hb.

We isolated the secondary fraction from normal adult Hb, by applying 1 ml 10% HbCO or HbO_2 to a 2½ × 15 cm column of Amberlite IRC-50 XE-64 equilibrated with citrate buffer pH 6.5 (0.20 g.ion Na^+/l). In normal adults the relative concentration of the minor fraction proved to be about 10% (for 4 samples the values found were 9.0, 11.7, 11.1 and 10.9% respectively). In some patients with anaemia a higher percentage (15–20%) was found. On rechromatography the Hb-a fraction retained its rapid rate of displacement, as compared with the main fraction; it showed no deviations from the main fraction as far as absorption of visible and U.V. light or denaturation velocity in alkaline solution were concerned. In paper electrophoresis it migrated somewhat more rapidly to the anode than the main fraction; in this respect and also in chromatography, it resembles the abnormal Hb-I very closely. Whether or not Hb-a and Hb-I are identical cannot be said as yet.

Somewhat different results were obtained by MORRISON AND COOK[55], on chromatographing 50 mg of HbO_2 on 0.9 × 50 cm Amberlite IRC-50 columns at 4°. The haemoglobin was eluted with phosphate–citrate buffer (pH 6.3) of constantly increasing ionic strength from 0.03 *M* to a maximum of 0.2 *M*; the flow rate was

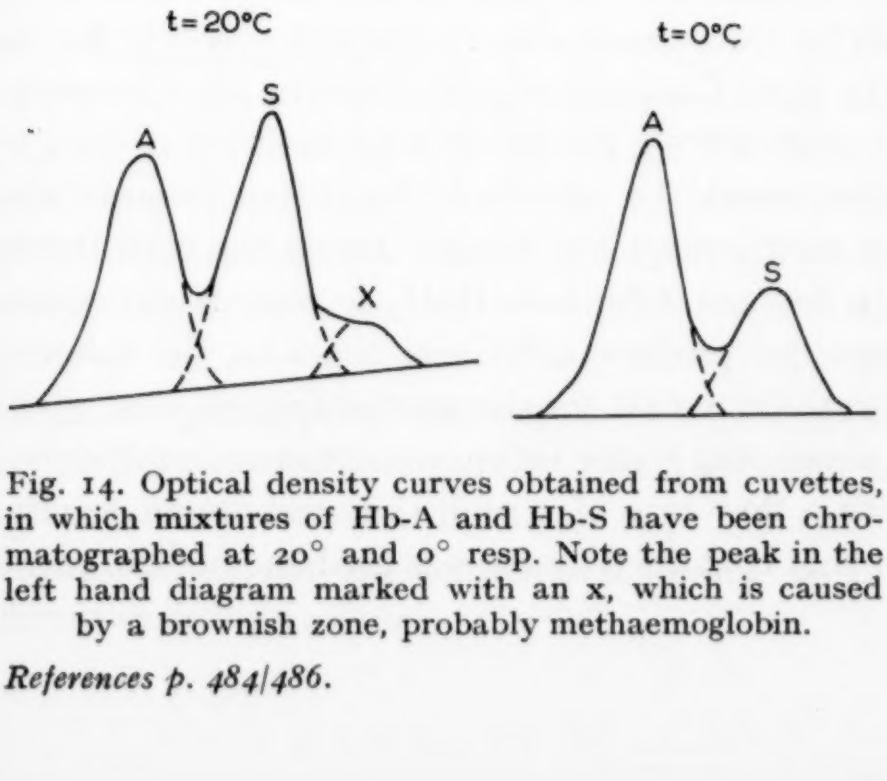


Fig. 14. Optical density curves obtained from cuvettes, in which mixtures of Hb-A and Hb-S have been chromatographed at 20° and 0° resp. Note the peak in the left hand diagram marked with an x, which is caused by a brownish zone, probably methaemoglobin.

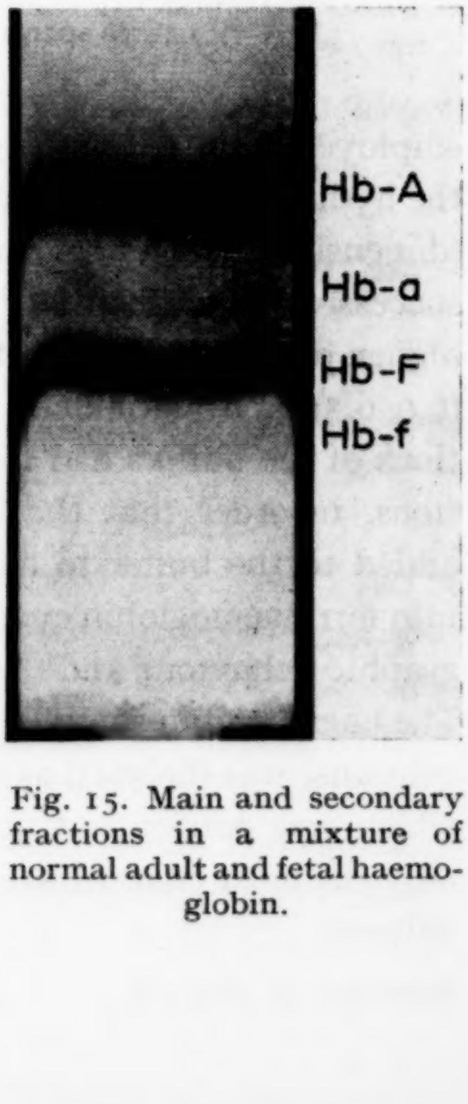


Fig. 15. Main and secondary fractions in a mixture of normal adult and fetal haemoglobin.

approximately 10 ml/h. With haemoglobin from normal adults, MORRISON AND COOK obtained 3 different fractions, the first representing about 10% of the total (possibly identical with the Hb-a component found by us[62]), the second and third constituting 84 and 6% of the total respectively. Each of the fractions gave an absorption spectrum typical for oxyhaemoglobin. The investigators do not report whether the different components were rechromatographed.

Operating under different conditions, ALLEN, SCHROEDER AND BALOG[2] investigated crystallized as well as uncrystallized normal human adult red cell haemolysates. Besides the main fraction (comprising about 90% of the total haemoglobin), they obtained various subfractions, which all exhibited a higher rate of displacement on the column than the major fraction. Under the conditions of the experiment HbO_2 and ferrihaemoglobin cyanide showed identical chromatographic behaviour. ALLEN *et al.* employed Amberlite IRC-50 of very uniform particle size: the resin was sifted wet in the hydrogen form and the fraction 200–250 mesh was used for preparing the columns (dimensions 1 × 35 cm for analytical work). The columns were employed for several successive experiments. For reasons of stability the elution buffers chosen were of higher pH (7.0–7.2) than those used by BOARDMAN AND PARTRIDGE (5.8–6.5), by us (6.0–6.5) or by MORRISON AND COOK (6.3). Consequently, the sodium ion concentrations of the buffers had to be lower (0.05–0.075 g.ion Na^+/l) than in earlier investigations, in order that the haemoglobin should be adsorbed. Potassium cyanide was added to the buffer to convert any methaemoglobin formed during the experiment into ferrihaemoglobin cyanide, which does not differ from HbO_2 as regards chromatographic behaviour and therefore does not produce additional zones on the column. The haemoglobin sample (containing 30–60 mg Hb for chromatography on 1 × 35 cm columns) was dialysed against the developing buffer before the chromatographic run, which was carried out at 5–6° with a flow rate of 5 ml/h. For rechromatography, only the most concentrated effluent that contained a zone was used and not the entire effluent.

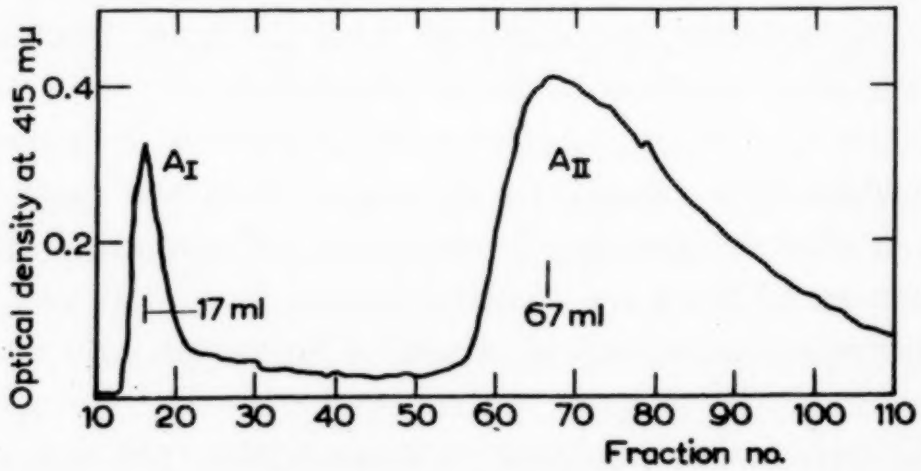


Fig. 16. Chromatogram of normal adult oxyhaemoglobin on a 1 × 35 cm column of IRC-50 Developer: phosphate buffer, containing 3.45 g $NaH_2PO_4 \cdot H_2O$, 2.66 g Na_2HPO_4 and 0.65 g KCN/l. The pH at 25° is 7.18 ± 0.02; the solution contains 0.0625 g.ions Na^+/l and 0.01 g.ions K^+/l. Temperature, 5–6°; rate of flow, 5 ml/h; fraction volume, 1 ml. The fractions were diluted to 3 ml before spectrophotometric reading. (ALLEN, SCHROEDER AND BALOG[2]).

In Fig. 16 a chromatogram of a fresh haemolysate of adult red cells is presented. Under the conditions of the experiment part of the haemoglobin (about 10%) is not adsorbed by the resin and can be easily and completely separated from the main fraction. No third component is visible. When the non-adsorbed fraction (designated A_I), was rechromatographed under somewhat different conditions (lower pH and ionic strength), 3 fractions (A_{Ia}, A_{Ib}, and A_{Ic}) were obtained besides some non-haem protein. Spectral studies revealed that during rechromatography some methaemoglobin cyanide had been formed.

Chromatography of cord blood oxyhaemoglobin with an elution buffer of pH 7.22 (0.075 g.ion Na^+/l) resulted, as in the first experiment with adult Hb, in a separation into two fractions: one was not adsorbed by the column and turned out to be foetal Hb, the other showed a rate of displacement identical with that of the main component in the experiment with adult Hb. Rechromatography of the foetal fraction with the same buffer that was used for the rechromatography of zone A_I, resulted in separation into two fractions: the first fraction eluted (designated F_I) constituted about 20% of the foetal Hb. Whether any subfraction of zone A_I is identical with a subfraction of zone F has not been established.

Table II gives some results of phenylalanine, leucine and isoleucine determinations in the different haemoglobin fractions prepared by ALLEN *et al.*

TABLE II

AMINO ACID ANALYSES OF SOME HAEMOGLOBIN PREPARATIONS AND FRACTIONS

(ALLEN *et al.*[2])

Preparation	*Ratios*	
	leu/phe	*ileu/leu*
Haemoglobin uncrystallized	1.9	0.014
Oxyhaemoglobin, crystallized	1.9	0.013
Carbonmonoxyhaemoglobin, crystallized	1.9	0.015
Oxyhaemoglobin, crystallized twice fractionally	1.9	0.017
Main peak of electrophorised haemoglobin	1.9	0.000
Trailing edge of electrophorised haemoglobin (including non-haem protein)	1.9	0.11
Zone A_{II}	1.9	0
Electrophorized zone A_I	1.8	0.037
Non-haem trailing edge of electrophorised zone A_I	1.4	0.34
Non-haem protein obtained by chromatography	1.6	0.46
Zone F_{II}	1.8	0.10
Zone A_{Ic}	2.0	0.044
Globin	1.8	0.013

ALLEN, SCHROEDER AND BALOG draw the following conclusions from their experiments:

(1) Both crystallized and uncrystallized adult haemoglobin contain, in addition to the main component, at least 3 haem proteins and 1 non-haem protein. The various components are not artifacts formed during preparation or chromatography; they differ in their isoleucine content.

(2) None of the haem proteins correspond to the slowly moving subfraction found by MORRISON AND COOK.

(3) None of the haem proteins could be definitely identified with foetal haemoglobin.

(4) Cord blood haemoglobin also contains minor components.

The authors make the following suggestion: "If the (chromatographic) methods were applied to the haemoglobin mixtures that are known to occur in certain pathological conditions, it probably would be possible to determine whether entirely different components are present or whether components normally present in minor amount have simply been increased proportionately".

Apart from the conclusions concerning the heterogeneity of adult red cell haemolysates, the investigations of ALLEN *et al.* are very important because they show that good preparative separations can be obtained in complex mixtures by chromatographing in several runs; this had already been suggested by BOARDMAN AND PARTRIDGE[8]. In the first run the mixture is divided into an adsorbed and an unadsorbed portion; in the following run or runs the non-adsorbed fraction is fractionated under slightly more acidic conditions and with a lower ionic strength. With this procedure there is less risk of denaturation occurring than with those in which all the components of a complex mixture are adsorbed and then desorbed one by one.

Adsorption on carboxymethyl cellulose

Ion-exchange adsorbents derived from cellulose which were introduced by PETERSON AND SOBER, are becoming more and more important in the chromatography of proteins, nucleoproteins and nucleic acids. PETERSON AND SOBER[60] prepared different types of cation and anion exchangers from cellulose powder, for instance by reaction with monochloracetic acid (product: carboxymethyl cellulose), with diethyl-2-chloroethyl-amine (product: diethylaminoethyl cellulose). The last-mentioned product, an anion exchanger, was first used by PETERSON *et al.*[78] in the chromatographic separation of serum proteins. Preliminary experiments gave promising results.

Recently, HUISMAN *et al.* employed the cation exchanger carboxymethyl cellulose in very successful experiments with different types of human haemoglobin[24]. The results of these experiments will be discussed in this section.

There are very marked differences between the cation exchangers Amberlite IRC-50 (discussed in the former section) and the carboxymethyl cellulose adsorbents.

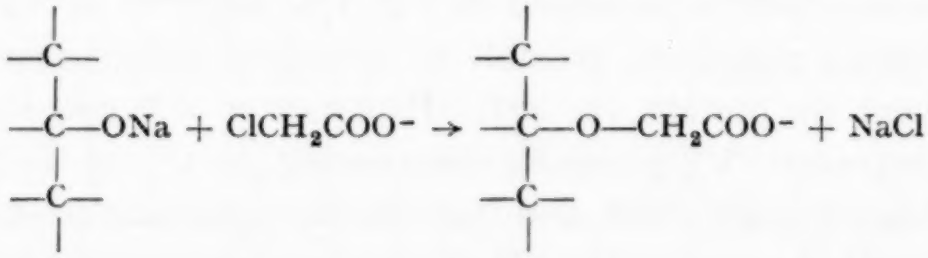
(1) To make sure that the carboxymethyl cellulose (CMC) will not be water-soluble in the sodium form, such reaction conditions are chosen for the preparation of the adsorbent that only few carboxyl groups are introduced (according to PETERSON AND SOBER CMC contains about 0.5–1.0 COOH/g dry weight; Amberlite IRC-50 contains about 10 mequiv. COOH/g dry weight).

Consequently, CMC adsorbent has a very low buffer capacity, so that it is suitable for gradient elution experiments; only the molarities and the pH of the elution buffers are of importance. A certain disadvantage of the low buffer capacity is the necessity to dialyse the protein sample very carefully against the first elution buffer before starting the chromatographic experiment. In chromatography on Amberlite IRC-50 this is not necessary. Column artifacts are easily formed as a result of the low buffer capacity of the cellulose adsorbent: when a protein solution is applied to a CMC column under such conditions that the protein is just adsorbed but a small increase in ionic strength of the buffer solution will cause desorption, it is often observed[23, 63] that the first portion of the protein sample is adsorbed at the top of the column; this adsorption, however, releases so many cations from that part of the column that the rest of the column is in equilibrium with a higher cation concentration, which results in non-adsorption of the last part of the protein sample applied to the column. In this way pseudo-heterogeneity can be observed in protein samples that are in fact homogeneous. The occurrence of such artifacts can be avoided by adsorbing under less critical conditions.

(2) Carboxymethyl cellulose can adsorb reversibly its own dry weight of protein, whereas Amberlite IRC-50 because of its high percentage of cross-linkages can only adsorb protein on the particle surface, that is a few milligrams of protein per gram dry weight. Adsorption on CMC is reversible, probably because the number of carboxyl groups per gram adsorbent is low. There are enough carboxyl groups present for adsorption to occur, but not so many that strong multipoint attachment will result in irreversible deformation. This is another feature that makes CMC more suitable for gradient elution chromatography than other adsorbents known so far. At the beginning of an experiment the various components of a complex mixture can be run on a column at such a rate that the R_F values are much lower than 0.5, without the risk of denaturation occurring caused by too strong adsorption; the components can then be desorbed one after another by slightly increasing the buffer concentration.

Apart from the differences mentioned here, the considerations of BOARDMAN AND PARTRIDGE concerning protein adsorption by Amberlite IRC-50 are qualitatively also valid for CMC (p. 459).

CMC is prepared by means of the reaction of Williamson:



$$-\mathrm{C}-\mathrm{ONa} + \mathrm{ClCH_2COO^-} \rightarrow -\mathrm{C}-\mathrm{O}-\mathrm{CH_2COO^-} + \mathrm{NaCl}$$

Procedure. According to PETERSON AND SOBER[60], a solution of 90 g NaOH in 200 ml water is stirred into 60 g of Whatman cellulose powder that has passed a 325 mesh sieve. The paste obtained is cooled in an ice bath for half an hour under occasional stirring*. A solution of 30 g monochloroacetic acid in 40 ml water is added slowly under mechanical stirring. While stirring is continued the beaker containing the reaction mixture is immersed for 20 min in a 70° oil or water bath, after which it is transferred to an ice bath. After cooling, 500 ml of 10% acetic acid is added slowly, also under mechanical stirring. The yellow reaction mixture, which is still alkaline, is then poured into 1 l of distilled water and allowed to settle for at least 3 h. The cloudy yellow supernatant is removed, the sediment is shaken or stirred with a fresh portion of distilled water and again allowed to settle. This washing procedure is continued until the suspension is no longer yellow. The acid form of the adsorbent is obtained by adding 10 ml glacial acetic acid to the washed suspension, filtering on a coarse sintered glass or Buchner funnel, washing first with water and then with ethanol and drying at room temperature *in vacuo*.

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* It is our experience that if an additional 100 ml of water is added the mass obtained can more easily be stirred.

SOBER AND PETERSON report a yield of 45 g of white powder, containing 0.7 mequiv. acidic groups per g*.

Columns of CMC are prepared in the same way as described for Amberlite IRC-50 (p. 462). They are also equilibrated with buffer by passing buffer through the column until the pH of the inflowing buffer and the effluent are exactly equal.

HUISMAN *et al.*[24] employed glass tubes (55 × 0.9 cm) filled up to a height of 52 cm with CMC. pH-gradient elution chromatography was performed as follows:

The column was first equilibrated with a 0.01 *M* phosphate buffer, pH 6.8 (sometimes pH 6.5, 6.3 or 6.0). The phosphate buffer was always a mixture of two solutions, containing 1.78 g $Na_2HPO_4 \cdot 2$ aq. per l, and 1.56 g NaH_2PO_4 per l, respectively. After the introduction of a solution of 100–150 mg of HbCO in a volume of 5 ml (the sample was first dialysed for 24 h against the starting buffer at 4°), the glass tube was filled to the top with the starting buffer, then connected with a mixing chamber (50 ml) which also contained the starting buffer. The mixing chamber was connected with a supply bottle containing 0.01 *M* phosphate buffer pH 8.0 (prepared in the same way as described for the starting buffer). A flow rate of 4–6 ml/h was maintained throughout each experiment; the temperature was kept below 10°.

By this procedure several excellent separations were obtained in various mixtures. The recovery was always higher than 97%.

Normal adult haemoglobin (preparation see p. 447). An elution diagram of normal adult red cell haemolysate is presented in Fig. 17a. After 25 ml have run through, the pH curve shows a maximum, possibly as a result of sodium ions being displaced from the adsorbent by protein (p. 471). HUISMAN *et al.*[24] suggest the possibility that the first component (V), generally constituting ½–1% of the total amount of haemoglobin, does not really exist, and that the corresponding peak is caused by the initial rise of the pH. As soon as the pH of the eluent is 7.40 ± 0.01, a fraction (A_1) (10% of the total amount of Hb) is eluted. This fraction is probably identical with the rapidly migrating subfraction obtained in Amberlite IRC-50 chromatography (p. 466). The main fraction A_0 is eluted at pH 7.49 ± 0.01, followed at pH 7.69 ± 0.01 by the last (A_2-fraction) peak, containing 0.5–2.0% of the total Hb; this last fraction is not seen in Amberlite chromatography. Paper electrophoresis on Whatman No. 3 MM paper at pH 8.6 (for description see p. 451) showed that fractions A_1 and A_2 behaved electrophoretically as Hb-J and Hb-E, respectively; A_1 migrated somewhat faster than Hb-A and A_2 much slower than this substance. The authors suggest that these subfractions may be identical with the electrophoretical subfractions obtained by KUNKEL AND WALLENIUS (p. 455).

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* We[63] obtained CMC batches containing respectively 0.46, 0.48 and 0.45 mequiv. acidic groups per g dry weight; possibly these lower values resulted from the dilution of the reaction mixture at the beginning of the procedure (see footnote*).

A chromatogram of a Hb sample of a patient with Hb-J trait reveals that the abnormal haemoglobin is eluted at almost the same pH (7.39) as Hb-A_1 (7.40). This suggests that the so-called abnormal Hb-J might be identical with a minor fraction (A_1) of normal adult haemoglobin. However, it should be borne in mind that by chromatography on Amberlite IRC-50 cuvettes the faster moving subfraction could be separated from the main fraction, whereas Hb-J did not separate from Hb-A. Thus the possibility exists that either the faster moving Amberlite-fraction Hb-a is not identical with the CMC-fraction A_1, or that the CMC-fraction Hb-A_1 is not identical with Hb-J. This can perhaps be checked by estimation of the amino acid composition of the different haemoglobin fractions.

Another possible identity is that of fraction A_2 and the abnormal haemoglobin E. Electrophoresis revealed no difference, and chromatography of a haemoglobin sample of Hb-E trait blood on CMC showed that Hb-E was eluted at the same pH (7.68) as Hb-A_2 (7.69) (Fig. 17c). It is very likely that the CMC-fraction Hb-A_2 is identical with the Hb-A_2 fraction obtained electrophoretically, for the following reasons: their electrophoretic mobilities are the same, the percentages present in normal adults are of the same order, and these percentages are raised to almost the same extent in patients with Cooley trait.

Cord blood haemoglobin. Fig. 18b is an elution diagram of cord blood haemoglobin

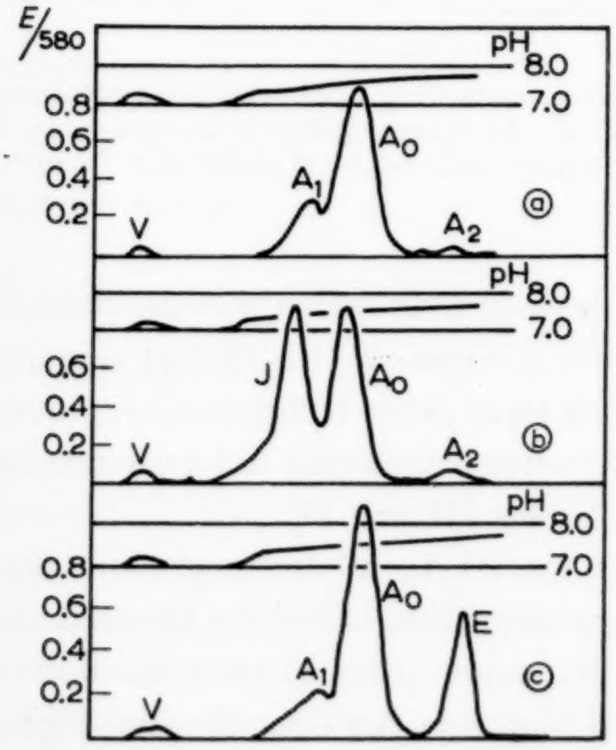

Fig. 17. pH-gradient elution diagrams of some haemoglobin samples chromatographed on CMC columns. (a) Normal adult HbCO; (b) HbCO from Hb-J trait; (c) HbCO from Hb-E trait. (HUISMAN, MARTIS AND DOZY[24]).

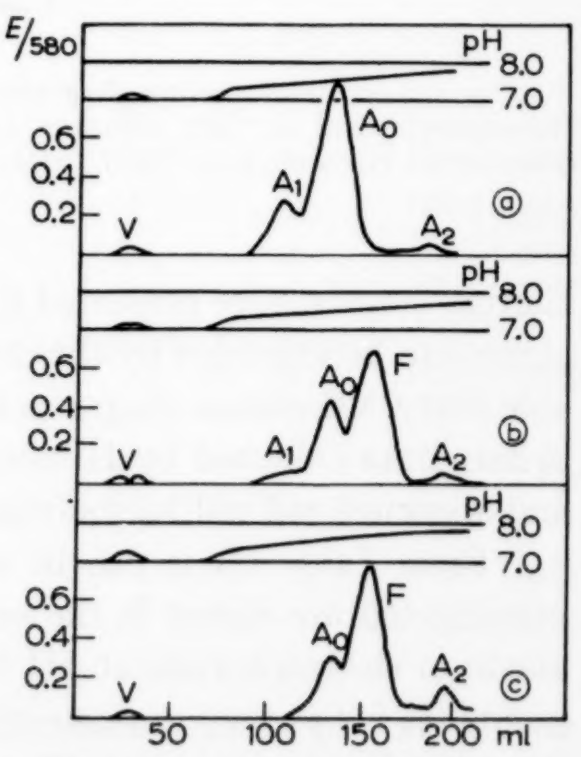

Fig. 18. Behaviour of foetal haemgolobin in pH-gradient CMC chromatography. (a) Normal adult HbCO; (b) HbCO from full term infant; (c) HbCO from three month old premature infant. (HUISMAN, MARTIS AND DOZY[24]).

of a normal full term infant; besides *one* well-separated Hb-F peak, all adult components A_1, A_0 and A_2 are present. These results differ in some respects from those of Allen *et al.* obtained with Amberlite IRC-50 chromatography. In the latter case foetal components were recognized and only one adult component. In special cases fraction A_1 is not observed in CMC chromatography, while fraction A_2 is then increased several fold (Fig. 18c).

It is further remarkable that Hb-F has a lower rate of displacement than Hb-A_0 on CMC columns. In Amberlite chromatography it moves faster than Hb-A.

Behaviour of some abnormal haemoglobins. Besides the investigation of Hb-J and Hb-E elution experiments were also carried out with mixtures containing the haemoglobins S, C, H, L, as well as a newly detected haemoglobin, designated Buginese X.

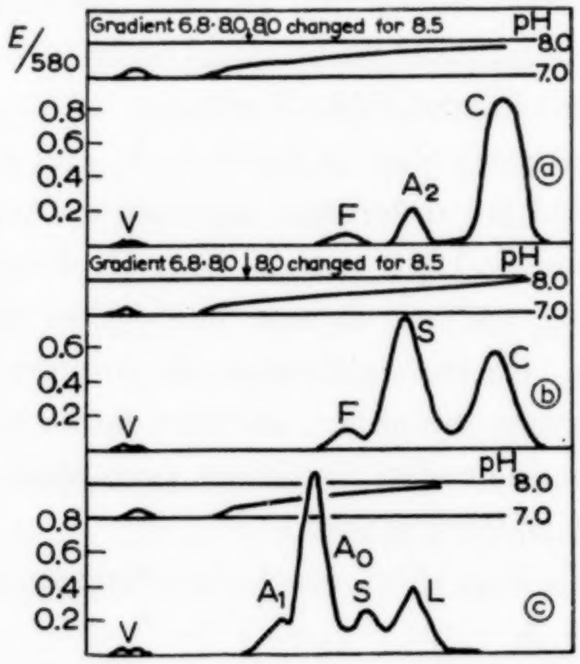

Fig. 19. pH-gradient elution diagrams of various mixtures containing abnormal haemoglobins, chromatographed on CMC columns. (a) Homozygous Hb-C; (b) Haemoglobin S–haemoglobin C disease; (c) Heterozygous Hb-L, after addition of some Hb-S. (Huisman, Martis and Dozy[24]).

Elution diagrams are presented in Fig. 19. It can be seen that each abnormal haemoglobin can be identified by the distinct pH at which it is eluted. It is further remarkable that CMC elution diagrams reveal more details than other techniques employed so far. Data collected by Huisman and co-workers when studying different normal and abnormal red cell haemolysates are listed in Tables III and IV.

From Table III it can be seen that except for Hb-L, all haemoglobin types investigated are eluted in the same order as their migration velocities towards the anode in electrophoresis at pH 8.6, and also in the same order as their iso-electric points (as far as they are known). The elution pH is always 0.4–0.6 pH units higher than the iso-electric point. The results of the pH-gradient elution experiments of Huisman *et al.* suggest that the separations are mainly caused by differences in electrostatic attraction at various pH values. As soon as a haemoglobin type has passed its iso-electric point, electrostatic repulsion forces very rapidly become more

TABLE III

BEHAVIOUR OF DIFFERENT HAEMOGLOBIN TYPES IN CMC CHROMATOGRAPHY AND ELECTROPHORESIS AT pH 8.6

	Hb type									
	H	*J*	A_1	A_0	*F*	*L*	*S*	*E*	A_2	*C*
Elution pH in CMC chromatography	6.7 — 6.9	7.39 ± 0.01	7.40 ± 0.01	7.49 ± 0.01	7.58 ± 0.01	7.71	7.64 ± 0.02	7.68 0.01	7.69 0.01	7.82
Migration velocity to the anode at pH 8.6	H >	J	>	A_0 >	F >	L >	S >	E =	A_2 >	C
Iso-electric point	6			6.87	6.98		7.09	7.20		7.30

TABLE IV

THE PERCENTAGE AMOUNT OF DIFFERENT HAEMOGLOBIN COMPONENTS IN VARIOUS RED CELL HAEMOLYSATES

Type of blood	*Number of cases*	*V*	A_1	A_0	A_2	*F*	*Abnormal component*
Normal adult	7	1.5 (1–2)	10.5 (8–15.5)	87 (84–90^5)	1 (0.5–2)	—	—
Cord blood	3	1 (0–1^5)	2 (0–2^5)	22 (19^5–25^5)	0.7 (0^5–1)	75 (69–79^5)	—
Cord blood (premature)	1	2	0	11	6	81	—
Cooley's trait	2	1 1.5	6.5 12	86.5 81.5	6 5	— —	— —
Cooley's anaemia	2	1 1	4 12.5	17 55.5	1 0.5	77 30.5	— —
Sickle-cell anaemia (after blood transf.)	1	0.5	4.5	21	5.5	7.5	Hb-S: 61%
Hom. Hb-C disease	1	0.5	—	—	8.5	3.5	Hb-C: 87.5%
Hb-S/Hb-C disease	1	0.5	—	—	?	5.5	Hb-S: 49.5% Hb-C: 44.5%
Hb-E trait	4	1 (0.5–2^5)	10 (8^5–11)	65.5 (62–71^5)	—	—	Hb-E: 22^5% (16–28.5)
Hb E thalassaemia	3	1 (0–1^5)	3^5 (0–6^5)	18 (10–30)	—	39 (30–52^5)	Hb-E: 38% (32^5–48)
Hb H disease	1	—	11	73	1	—	Hb-H: 15%
Hb J trait	1	1	?	45.5	2	—	Hb-J: 51.5%
Hb L trait	1	0.5	9.5	65	?	—	Hb-L: 25%

important than the short range forces, which hold the haemoglobin, now negatively charged, to the also negatively charged adsorbent.

It would be interesting to perform chromatographic experiments with haemoglobin on CMC columns, working with a buffer concentration gradient at a fixed pH (for instance pH 6 or 6.5 as in the chromatography on Amberlite IRC-50 columns).

At these low pH values, secondary range forces will perhaps become relatively more important, and differences in structure rather than differences in electric charge will be reflected in the elution diagrams.

On comparing pH gradient elution on CMC columns with chromatography on Amberlite columns, we observed that, in general, elution curves of various mixtures of haemoglobins adsorbed by CMC show more detail than chromatography of the same mixtures on Amberlite at a fixed pH. Moreover, every component is eluted at a highly reproducible pH value; this value is a characteristic of a certain haemoglobin component. However, it should be borne in mind that although the values were reproducible for the adsorbent prepared by HUISMAN *et al.*, it is not yet known whether deviating elution pH values will be found on columns prepared from other batches of carboxymethyl cellulose. On the other hand the characteristics of the haemoglobin types in Amberlite chromatography (elution volumes and rates of displacement at fixed pH) are much less reproducible, for they are highly dependent on particle size, rate of flow, etc.; only values relative to those of normal adult haemoglobin (A_0) can be obtained with reasonable reproducibility.

Carboxymethyl cellulose is also more suitable for preparative separation of the various haemoglobin components from mixtures, because of its higher (reversible) adsorption capacity. In one case, however, Amberlite IRC-50 is to be preferred, namely in cuvette chromatography, where the different haemoglobin types are recognized by visible separation on the column. Because of the high buffering capacity of the resin (which in gradient elution chromatography is a disadvantage), a pH and cation concentration gradient can be maintained on the column for several hours, simply by passing a buffer solution of constant pH and ionic strength through the resin column, which was originally in the acid form. With this procedure haemoglobin types can be seen as sharp, distinct, zones, moving with different, but very low, rates of displacement.

Comparing chromatographic techniques with other methods employed in haemoglobin differentiation, it can be said that, in general, chromatographic elution curves show much detail, certainly more than electropherograms and at least as much as salting-out diagrams. The advantage of elution chromatography over the salting-out procedure of DERRIEN *et al.* lies in the fact that the different components obtained in the elution procedure are fairly pure and not denatured, and therefore ready for further characterization.

As a method for quantitative estimation, chromatography is comparable to electrophoresis, cuvette chromatography to paper electrophoresis, and CMC elution curves to moving-boundary diagrams. As a routine method, chromatography is more laborious than electrophoresis, especially paper electrophoresis. However, as regards simplicity the cuvette technique comes very close to paper electrophoresis. Paper electrophoresis (or zone electrophoresis on cellulose acetate strips) and the chromatographic cuvette technique as well as a combination of the methods are very suitable for the detection of abnormal haemoglobin in clinical routine analysis, as will be shown in the next part of this review.

III. THE DETECTION AND CHARACTERIZATION OF ABNORMAL HUMAN HAEMOGLOBINS BY COMBINING DIFFERENT TECHNIQUES

1. *Characteristics of different types of human haemoglobin*

Normal adult haemoglobin

Moving-boundary electrophoretic patterns in general contain one sharp peak; the cathodic mobility in 0.1 *M* phosphate buffer, pH 6.5, is $2.4 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}; iso-electric point in 0.1 *M* phosphate buffer at pH 6.87.

In *zone electrophoresis* at pH 8.6 on paper or cellulose acetate, or in starch, the main fraction has a mobility similar to that of the β_2-globulin fraction of human serum. In starch electrophoresis, besides the main fraction (A_0) two minor components are seen: one (A_1) migrates slightly more rapidly than A_0, the other (A_2, about 2–3% of the total haemoglobin) moves more slowly and separation from the main fraction is good in starch as well as on cellulose acetate strips. The amount of this A_2 fraction is generally increased in thalassaemia trait to about twice the normal value.

In *column chromatography* on Amberlite IRC-50 besides the main fraction another component is seen, which moves a little more rapidly, this component (designated Hb-a or Hb-AI) represents about 10% of the total haemoglobin.

pH-gradient elution chromatography on carboxymethyl cellulose gives rise to three peaks: the first (A_1, 10%) has an elution pH of 7.40, the second and main fraction (A_0) has an elution pH of 7.49 and the third fraction (A_2, 1%) is eluted at pH 7.69. The A_2 level is elevated in thalassaemia trait.

The chromatographic fractions A_1, A_0 and A_2 seem to be identical with the corresponding electrophoretical zones A_1, A_0 and A_2, but this has not yet been proved.

Adult oxy-haemoglobin is very rapidly denatured in the *alkali-denaturation test*. Its "half-life" in 0.05 *N* NaOH solution is about 4 sec[17].

Solubility. In the salting-out method of DERRIEN *et al.*[67] (see p. 456) HbCO-A is precipitated in the range from about 77 to 89 vol. % of 3.5 *M* phosphate[9, 27, 67]. Deoxygenated Hb-A is precipitated in the range 72–84%[27]. The solubility of deoxygenated Hb-A in 2.58 *M* (= 74% of 3.5 *M*) phosphate solution at 25° is 1.39 ± 0.15 g/l as estimated by ITANO[36].

From experiments with dinitrophenyl-globin RHINESMITH, SCHROEDER AND PAULING[64] concluded that there are 4 N-terminal amino acid residues in the haemoglobin molecule, all valyl residues.

Foetal haemoglobin (*Hb-F*)[7]

In *moving-boundary electrophoresis* in 0.1 *M* phosphate buffer, pH 6.5, Hb-F does not separate from Hb-A. Separations are only possible in special buffer solutions, for instance phosphate buffer pH 6.8, ionic strength 0.01[3, 71]. The iso-electric point in 0.1 *M* phosphate buffer is at 6.98 (Hb-A: 6.87).

In *zone electrophoresis* at pH 8.6, Hb-F moves slightly less rapidly towards the anode than Hb-A_0. Mixtures of Hb-A and Hb-F behave as a single spot, migrating with intermediate velocity.

In *column chromatography* on Amberlite IRC-50, foetal haemoglobin is more weakly adsorbed than adult haemoglobin. In the cuvette method it is displaced 1.3 times more rapidly than Hb-A. In Amberlite chromatography a subfraction is also seen (Hb-f or Hb-F_I), which moves ahead of the main fraction[2, 55, 62]. In pH-gradient elution chromatography on CMC, Hb-F is eluted a little later than Hb-A_0. The elution pH of Hb-F is 7.58 (Hb-A_0: 7.49).

In the *alkaline denaturation test* HbO_2-F is much more resistant than Hb-A, its "half-life" in 0.05 N NaOH solution is about 20 minutes[6]. The alkaline denaturation technique is the best quantitative estimation method for Hb-F.

Absorption spectrum: In the case of Hb-F the so-called "tryptophan absorption maximum" is located at 289.8 mμ, whereas for all other haemoglobins known at present it is situated at 291 mμ. By means of this spectral speciality, isolated HbCO- or HbO_2-F fractions can very easily be identified[3, 43].

In the DERRIEN *salting-out method* HbCO-F is precipitated in the range from 88–95 vol. % of 3.5 M phosphate solution, pH 6.5 (HbCO-A: 77–89%). For deoxygenated Hb-F, the range is also a few % higher than normal. The solubility of deoxygenated cord blood haemoglobin in 2.58 M phosphate solution is 2.2 ± 0.2 g/l as estimated by ITANO[36].

The *amino acid composition of Hb-F* differs markedly from that of Hb-A, as estimated by different investigators[26, 72, 73]. A very typical difference is found in the isoleucine content: Hb-A contains 0.32 g of isoleucine per 100 g of haemoglobin, whereas Hb-F contains 1.83 g per 100 g. From hydrolysis experiments with DNP-globin, SCHROEDER AND MATSUDA[74] conclude, that foetal haemoglobin contains 2 N-terminal valyl residues instead of the 4 valyl residues found in normal adult haemoglobin.

Sickle-cell haemoglobin (*Hb-S*)[58]

Good separation from Hb-A is obtained in *moving-boundary electrophoresis* in 0.1 M phosphate buffer, pH 6.5, mobility: $2.9 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}.

In *zone electrophoresis* separation of Hb-S from Hb-A and Hb-F is also good. It migrates more slowly than the latter components. In electrophoresis in starch or on cellulose acetate strips it moves slightly more rapidly than the Hb-A_2 component.

In the *cuvette method with Amberlite IRC-50* the rate of displacement of Hb-S is 0.7 times that of Hb-A. Separation from Hb-A is good.

In *pH-gradient elution chromatography* on CMC its elution pH is found to be 7.64 (between that of Hb-F and Hb-A_2).

A very characteristic property of sickle-cell haemoglobin is its low *solubility* in the deoxygenated form. In this form it is precipitated in the salting-out method[27] within the range 52–66 vol.% of 3.5 M phosphate solution, whereas the precipitation range of the CO-form is almost normal (75–84%). The amino acid composition of Hb-S is almost identical with that of Hb-A[26, 73]. INGRAM, using a combination of paper electrophoretic and chromatographic techniques in the investigation of tryptic digestion products of Hb-S and Hb-A concluded that at two places in Hb-S there are valyl residues present instead of the glutamic acid residues found in Hb-A[32].

Haemoglobin C[34]

In *moving-boundary electrophoresis* in 0.1 *M* phosphate buffer, pH 6.5, the mobility of Hb-C has the highest value so far found for human haemoglobin: $3.2 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}. The iso-electric point in 0.1 *M* phosphate buffer is 7.30.

In *zone electrophoresis* at pH 8.6 as well as in *chromatography on Amberlite IRC-50*, Hb-C has the lowest rate of migration of all hitherto known haemoglobin types. In the cuvette method it is displaced 0.3 times as fast as Hb-A. Its elution pH in *CMC chromatography* is very high: 7.82. The *precipitation range* of HbCO-C in phosphate solution pH 6.5 is 68–76 vol. % of 3.5 *M* solution, as estimated by HUISMAN *et al.*[27], DERRIEN *et al.* have reported much higher values (83–93%). This difference in results has not yet been explained. According to HUISMAN *et al.*[27] the precipitation range of deoxygenated haemoglobin C falls almost within the normal range. On the other hand, ITANO reports a solubility (3.46 ± 0.41 g/l) in 2.58 *M* phosphate buffer higher than that of Hb-A (1.39 ± 0.15 g/l).

Using the technique mentioned above, INGRAM estimated that Hb-C differs chemically from Hb-A at the same two sites of the molecule as Hb-S; in Hb-C the glutamic acid residues of Hb-A are replaced by lysine residues.

Haemoglobin D[35]

The *electrophoretical properties* of Hb-D are identical with those of sickle-cell haemoglobin (the same mobility in buffer solutions of various kinds and pH's, the same iso-electric point).

In *cuvette chromatography* on Amberlite IRC-50, Hb-D could also not be distinguished from Hb-S.

A characteristic difference between Hb-S and Hb-D is found in the *solubility*, the deoxygenated form of the latter has a precipitation range in phosphate solution of the same order as that of Hb-A[9], whereas deoxygenated Hb-S is precipitated at much lower phosphate concentrations. For distinguishing between Hb-S and Hb-D, the simplified solubility method of ITANO[36] (see p. 458) is very useful.

Haemoglobin E[37]

Electrophoretical characteristics. The mobility in 0.1 *M* phosphate buffer, pH 6.5, is between that of Hb-A and Hb-S, the iso-electric point in 0.1 *M* phosphate buffer is at pH 7.20 (between Hb-S and Hb-C), the mobility in barbital buffer pH 8.6 is also between that of Hb-S and Hb-C, the pH–mobility curves of Hb-S and Hb-E thus cross each other at a pH between 6.5 and 7.2. Electrophoretically Hb-E cannot be distinguished from the normal A_2-fraction.

Chromatography. On Amberlite IRC-50 columns at pH about 6, Hb-E has a rate of displacement between those of Hb-A and Hb-S, it cannot be well separated from

Hb-A or Hb-S in this way. In pH-gradient elution on CMC columns, the elution pH of Hb-E is 7.68, similar to that of the Hb-A_2 fraction (7.69) and between those of Hb-S and Hb-C.

Amino acid analysis, as performed by STEIN *et al.*[79] revealed no marked differences between Hb-E and Hb-A.

Haemoglobin G[14]

In *electrophoresis* the pH–mobility curve lies between that of Hb-A and Hb-S. In 0.1 *M* cacodylate buffer pH 6.5, Hb-G separates well from Hb-A, no good separation of Hb-G and Hb-S can be obtained under these conditions. In barbital buffer pH 8.6, Hb-G separates well from Hb-S, but very poorly from Hb-A.

In Amberlite *chromatography* with the cuvette method, Hb-G is not separated from Hb-A, although it has a somewhat lower rate of displacement.

Solubility. Deoxygenated Hb-G is more soluble (5 g/l) than Hb-S (0.3 g/l) in 2.24 *M* (= 64% of 3.5 *M*) phosphate solution, but less soluble (0.6 g/l) than deoxygenated Hb-A (1.4 g/l) in 2.58 *M* (= 74% of 3.5 *M*) phosphate solution.

Haemoglobin H[65]

Electrophoretical data. The iso-electric point lies between pH 5.5 and 5.65, thus in contrast to the other Hb types, at pH 6.5 it moves towards the anode (mobility $-1.5 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}, differing considerably from that of Hb-A: $2.4 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}). At pH 8.6 in 0.1 *M* barbital buffer the difference is much smaller; the mobilities of haemoglobins H and A are then -3.8 and $-3.0 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1} respectively.

In *Amberlite chromatography*, Hb-H has the highest rate of displacement of the haemoglobins known so far. It separates well from Hb-F[25] which is also fast-moving.

In pH-gradient *CMC chromatography* Hb-H has the lowest elution pH: 6.7–6.9[24].

In the *solubility test* according to ITANO, deoxygenated Hb-H is practically insoluble in 2.24 *M* phosphate solution; after precipitation it cannot be redissolved by dilution of the buffer and oxygenation. Hb-H is also unstable in aqueous solution: a brownish flocculent precipitate is formed on standing.

The *amino acid composition* of Hb-H is different from that of Hb-A; it resembles that of Hb-F[29].

Haemoglobin I[69]

The *pH–mobility curve* and thus also the iso-electric point of Hb-I lie between those of Hb-H and Hb-A. The mobility in 0.1 *M* cacodylate buffer pH 6.5, is $1.7 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}. The difference in the mobilities of Hb-A and Hb-I (0.6–$0.7 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}) remains practically constant throughout the whole pH–mobility curve. Consequently Hb-I can be well separated from both Hb-A and Hb-H by electrophoresis at pH 6.5, but it is indistinguishable from Hb-H in paper electrophoresis at pH 8.6.

In *Amberlite chromatography*, Hb-I has a rate of displacement between those of Hb-A and Hb-F; mixtures of Hb-A and Hb-I form two well-separated zones[25].

Haemoglobin J[81]

Between pH 6.5 and pH 9.8, the *pH–mobility curve* of Hb-J lies between those of Hb-A and Hb-J. The difference in mobility between Hb-A and Hb-J is constant in this interval and is $0.3–0.4 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}. In paper electrophoresis in 0.025 *M* barbital buffer pH 8.6, a good separation of Hb-J and Hb-A can be achieved.

In *Amberlite IRC-50 chromatography* mixtures of Hb-A and Hb-J could not be separated[22]. In pH-gradient elution *chromatography on CMC*, Hb-J behaves very similar to the chromatographic fraction A_1. The elution pH of Hb-J is 7.39 ± 0.01, that of Hb-A_1 is 7.40 ± 0.01[24].

A mixture of deoxygenated Hb-A and Hb-J is significantly more soluble in 2.58 *M* phosphate buffer than Hb-A alone (2.00 ± 0.24 and 1.39 ± 0.15 g/l resp.).

Haemoglobin K[9]

In 0.025 *M* barbital buffer, the *electrophoretic mobility* of Hb-K is somewhat higher than that of Hb-A, but lower than that of Hb-J. It cannot be separated from Hb-A in paper electrophoresis, but a difference can be seen between spots obtained from normal Hb and those from mixtures of Hb-A and Hb-K.

In *chromatography on Amberlite IRC-50*, no difference between Hb-A and Hb-K can be observed.

In the *salting-out method* of DERRIEN *et al.*[68], HbCO–AK-mixtures give curves that are identical with those of HbCO–AD and AS-mixtures[9].

Haemoglobin L[1]

By means of *electrophoretical methods* Hb-L can only be separated from Hb-A at alkaline pH. In starch electrophoresis at pH 8.6, the mobility of Hb-L lies between those of Hb-A and Hb-G.

In *chromatography on Amberlite IRC-50* Hb-L forms a zone, moving with a rate of displacement between those of Hb-S and Hb-C, thus very different from Hb-A. In pH-gradient elution *chromatography on CMC*, the elution pH of Hb-L (7.71) also lies between those of Hb-S and Hb-C.

The *solubility* of Hb-L in phosphate solutions is reported to be normal.

Fig. 20 presents a schematic diagram of the properties of various haemoglobin types in electrophoresis and in Amberlite chromatography. It can be seen from this diagram that the two techniques complement each other in many cases, for instance, where electrophoresis at pH 8.6 fails to distinguish between adult and foetal Hb, or between Hb-G and Hb-K, or between Hb-H and Hb-I, Amberlite chromatography gives very good resolutions. On the other hand, where Amberlite chromatography gives no good separations (Hb-A and Hb-J, Hb-A and Hb-E), these can be achieved by electrophoresis. The combination cuvette chromatography + paper electrophoresis fails to distinguish between Hb-S and Hb-D, both haemoglobins behave identically in electrophoresis as well as in chromatography on Amberlite IRC-50. In this case resort must be made to solubility estimations. In all other cases so far known, paper electrophoresis in combination with chromatography on Amberlite cuvettes leads to a qualitative and sometimes a semi-quantitative analysis of an unknown haemoglobin mixture, provided the various fractions are present in amounts above 5%. An abnormal fraction, once detected, can be further characterized by the application of alkali denaturation, solubility studies, chromatography on carboxymethyl cellulose, mobility studies in moving-boundary electrophoresis, estimation of amino acid composition.

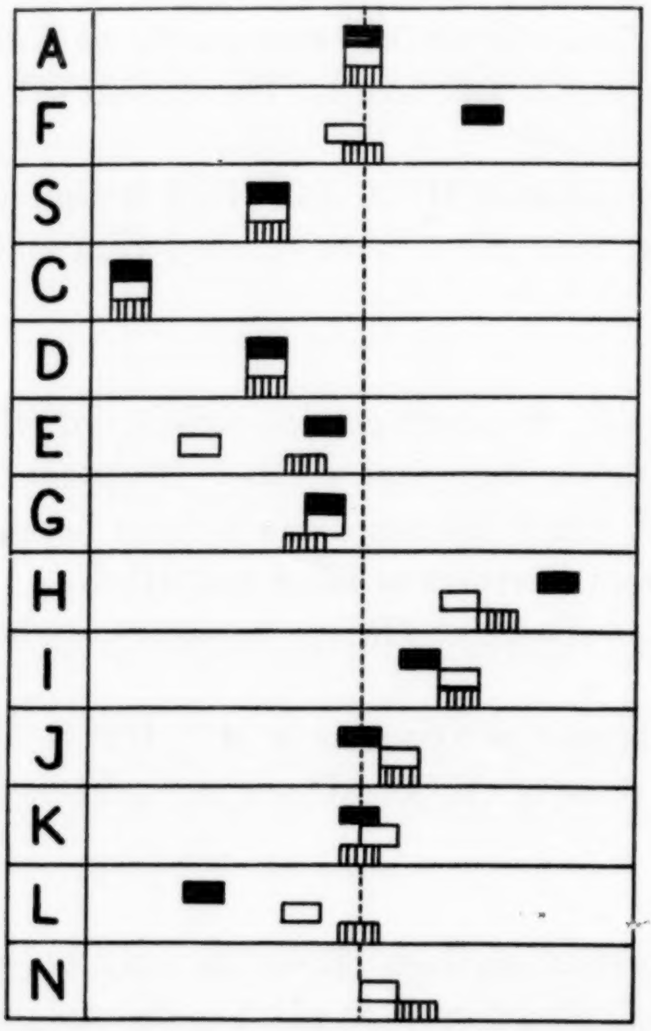

Fig. 20. Schematic comparison of the relative mobilities of different types of human haemoglobin in: Zone electrophoresis at pH 8.6 (□ —→ +), Moving-boundary electrophoresis at pH 6.5 (▥ —← +), Chromatography on IRC-50 at pH about 6.5 (■ →).

The application of different techniques is illustrated in the following description of a study of haemoglobins in the case of a family consisting of father, mother, two sons and three daughters[31]:

The haemoglobin of the mother (indicated as Mrs. W) behaved in cuvette chromatography as a tailing zone, with a rate of displacement corresponding to that of normal adult Hb. In paper electrophoresis at pH 8.6 on Whatman No. 3 MM paper, two well-separated zones were observed, one with a velocity equal to that of Hb-A, the other with a velocity somewhere between those of Hb-S and C.

These results indicated the presence of Hb-E besides Hb-A. Additional experiments revealed, that no alkali-resistant fraction was present; moving-boundary electrophoresis showed two components, one (62%) with a mobility similar to that of Hb-A, the other (38%) with a mobility similar to that of Hb-E.

The haemoglobin of the father showed no abnormality in paper electrophoresis; in cuvette chromatography a small zone was observed (about 10% of the total Hb) with a rate of displacement similar to that of Hb-F. Alkali-denaturation velocity measurements indicated that there was 11% of an alkali-resistant fraction. Thus, the blood of the father contained besides normal adult Hb, 11% of foetal haemoglobin, as in many cases of thalassaemia trait.

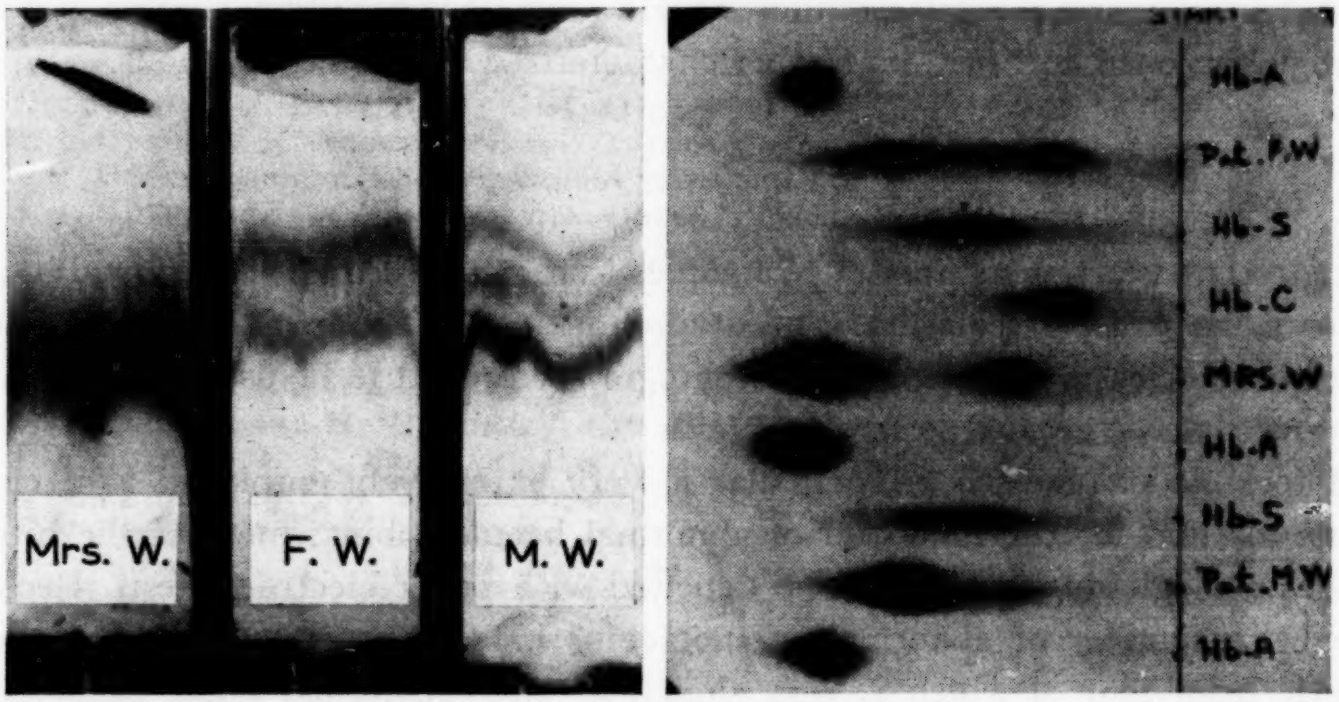

Fig. 21. A combination of cuvette chromatography and zone electrophoresis at pH 8.6 in a study of a family, in which Hb-E as well as thalassaemia occur.

Two children exhibited the same haemoglobin pattern as that of the mother, another child showed that of the father.

The fourth child (designated pat. F. W.), a boy of five years, had severe anaemia. Electrophoretic and chromatographic haemoglobin patterns are shown in Fig. 21. In the chromatogram, two zones can be seen, one (58%) with an almost normal velocity, the other (42%) with a velocity like that of foetal Hb. In the electrophoretic pattern, an F-like and an E-like zone were observed. Alkaline-denaturation velocity estimations revealed that 43% alkali-resistant Hb was present, moving-boundary electrophoresis showed that besides a resembling Hb-A component (62%), 38% Hb-E was present. From the results obtained with chromatography and alkaline denaturation it seemed very probable that 42% of the component that resembled Hb-A in electrophoresis, consisted of Hb-F, therefore it was assumed, that 20% of the total Hb consisted of Hb-A. Cuvette chromatography with about 5 mg HbCO and with slow elution velocity resulted in a chromatogram containing three zones, very probably representing Hb-F, Hb-A and Hb-E (in order of decreasing rate of displacement) (Fig. 13, 3, p. 446). The child is an example of a patient suffering from heterozygous thalassaemia Hb-E disease. The fifth child (pat. M. W.) was seven months old when examined. Her blood still contained 71% of alkali-resistant haemoglobin, the rest was probably a mixture of Hb-A and Hb-E (see Fig. 21 for the chromatogram and electrophoretic pattern). This child is very probably also heterozygous for thalassaemia and Hb-E.

This family study shows that also in more complicated cases, the abnormal Hb-fractions can be detected and provisionally identified by a routine combination of chromatography and zone electrophoresis. However, it is necessary that an abnormal haemoglobin, once detected, be further characterized with other methods.

2. *Conclusive remarks*

During recent years, a number of techniques that can be applied on an analytical as well as a preparative scale, have been adopted for the study of abnormal haemoglobins. Zone electrophoresis and chromatography proved to be useful, because both techniques can be applied in mass surveys.

Paper electrophoresis in particular is very extensively employed as a clinical routine method for the detection of abnormal haemoglobins; most of the hitherto known haemoglobins can indeed be detected with paper electrophoresis. Recently, however, a number of abnormal haemoglobins have been discovered, the electrophoretic behaviour of which is very similar to that of normal adult haemoglobin. These abnormal haemoglobins are very easily overlooked if only one technique is applied. Therefore it is suggested that a combination of at least two techniques should invariably be used for the detection of abnormal haemoglobins.

The techniques must be improved because of the growing importance of the evaluation of the so-called "sub-fractions" (in the diagnosis of thalassaemia trait).

It is to be expected that the replacement of the protein-adsorbing paper strips in zone electrophoresis by non-adsorbing material, such as starch and cellulose acetate, and that the introduction of cellulose ion-exchangers in chromatography will contribute much to making the analyses of haemoglobin mixtures more sensitive. Such sensitive methods of analysis are necessary, because further investigations can only be of importance in the study of heredity and haemoglobin synthesis, if performed on very clearly defined haemoglobin samples.

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THE DETECTION OF PHOSPHATES ON CHROMATOGRAMS

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A number of procedures have been described for the detection of organic phosphates on paper chromatograms. Most methods involve the use of an acid molybdate reagent, with subsequent reduction by one of a variety of reducing agents (*cf.* Hettler[1]). The most commonly employed are those of Hanes and Isherwood[2], where the reducing agent is hydrogen sulfide, and of Bandurski and Axelrod[3], where reduction is accomplished by ultraviolet irradiation. A procedure for the detection of phosphate spots on chromatograms without the use of molybdic acid has been described by Wade and Morgan[4].

The present communication deals with an alternative method for the chemical reduction of phosphomolybdate spots on paper; the method has proved satisfactory for the detection of a variety of phosphate compounds.

EXPERIMENTAL

Reagents

(1) *Molybdate reagent*:

5% (w/v) ammonium molybdate solution	3 ml
5 *N* HCl	7 ml
acetone	to 100 ml.

This reagent should not be kept for longer than one week.

(2) *Stock vanadyl chloride solution.* The solution was prepared by dissolving 2 g of vanadium pentoxide, V_2O_5, in approximately 20 ml of boiling conc. HCl; boiling was continued for 10 min after the colour changed to blue-green. The volume was then adjusted to 37 ml by the addition of 6 *N* HCl, and the solution diluted with water to 400 ml. This stock solution, which keeps indefinitely, thus contains 0.5% of V_2O_5 in approximately 0.5 *N* HCl.

(3) *Reducing reagent*:

Stock vanadyl chloride solution	20 ml
acetone	to 100 ml.

Approximately 250 mg of fine zinc powder is added to the green coloured reducing reagent and the mixture shaken in a stoppered flask until the solution is quite clear and brown in colour (about 10–20 sec). At this stage the zinc powder settles as a

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spongy precipitate and the clear reagent is recovered by decantation. This reagent should be prepared immediately before use.

Procedure for paper chromatograms

After development, the papers are thoroughly dried and then dipped in the molybdate reagent and allowed to dry in the air at room temperature, or in a stream of warm air. When the acetone has evaporated the papers are dipped in the reducing reagent and again allowed to dry in air. Blue spots are produced in about 2 min on a white background (an immediate blue background colour is produced, but this fades in 2 min). The background remains white for about 24 hours, after which period it slowly turns blue. If desired, the white background may be restored by dipping the chromatograms in a mixture of 1 part of 5 N HCl and 9 parts of acetone. The chromatogram is then allowed to dry in the air, whereupon the background fades slowly, leaving the spots unaffected.

Several papers in succession may be dipped in the reducing reagent, but it is ineffective once its colour has reverted to green. However, it may again be reduced by shaking with Zn powder as before.

The procedure described above was applied to a number of chromatograms. The following spots were detected after evaporation of the molybdate reagent at room temperature and subsequent reduction:

Inorganic orthophosphate (blue); pyrophosphate (purple); phosphocreatine (blue); ethanolamine phosphate (blue-green); adenosine triphosphate and adenosine diphosphate (faint blue). Adenosine monophosphate and glycerophosphate were not detected. Heat treatment of the paper (*cf.* ref.[2]) in the presence of the molybdate reagent would be required to hydrolyze the more stable esters.

Sensitivity

Since orthophosphate is probably required for the formation of the coloured complex, the sensitivity of the method was determined using successive dilutions of a solution of KH_2PO_4. The lower limit of detection for orthophosphate was found to be 0.02 μg/cm^2.

DISCUSSION

The method described above has been found convenient and rapid in use. The sensitivity limits obtained compare favourably with the other methods (*cf.* Bandurski and Axelrod[3] – 0.1 μg P). There is no critical exposure time at any stage, nor is there any need for rigorous moisture control. The background is completely clear at least within 24 hours of development. The present modification involves the reduction step only; the existing hydrolytic procedures which liberate orthophosphate from the more stable compounds can be applied prior to reduction.

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SUMMARY

A new method, involving reduced vanadyl chloride in acetone for the identification of phosphates on paper, has been described.

The main advantages of the method are rapidity of use, colourless background and high sensitivity (0.02 μg P/cm^2).

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THE DETERMINATION OF ACTIVITY COEFFICIENTS AT INFINITE DILUTION FROM GAS CHROMATOGRAPHIC MEASUREMENTS

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INTRODUCTION

A number of recent papers[1–8] have shown the usefulness of gas chromatography in the study of the thermodynamics of systems involving volatile solutes and involatile solvents. PORTER *et al.*[7, 8], ANDERSON AND NAPIER[1] and KWANTES AND RIJNDERS[5] have concluded that (except for polar solutes in non-polar solvents) activity coefficients at infinite dilution (γ°) derived from gas–liquid chromatographic measurements are sound and do not depend upon operational factors, *e.g.* the nature of the support, the amount of solvent, and the flow-rate. For polar solutes in non-polar solvents the polar properties of the solid support can influence the retention time of the solute; this can be overcome by using a non-polar support such as metal helices[5].

KEULEMANS[4] has recently reviewed much of the above work, suggested methods for the calculation of γ°, and tabulated values of γ° for a number of hydrocarbons and oxygenated compounds. A satisfactory agreement was found[4, 5] between values of γ° calculated from (i) chromatographic data for *n*-paraffinic hydrocarbons in up to *n*-C_{16} paraffinic solvents, (ii) the relation of BRÖNSTED AND KOEFOED[9] and (iii) with those found by VAN DER WAALS[10]. However, many of the above values from chromatographic data were not regarded as highly accurate[4] since they were derived from incidental analyses carried out for other purposes. A greater accuracy can easily be achieved in experiments designed for that purpose and the necessary control and measurement of operating parameters will be considered in this paper.

In previous qualitative and quantitative analyses of halogenated hydrocarbons by gas–liquid chromatography[11, 12] a large number of retention volumes were obtained, from which activity coefficients have now been calculated. These are not claimed to be highly accurate, but are useful for comparison purposes since no other thermodynamic data for these substances have been calculated from gas chromatographic measurements.

EXPERIMENTAL

The apparatus and general procedure have been described previously[11, 12]. Retention times were measured from the time of injection of the sample to the time of appearance of the peak maxima on the recorder chart, and were corrected for the "dead-time" of the apparatus (measured by injection of a small sample of either hydrogen or helium). The flow-rate was measured with a soap-film flow-meter at atmospheric pressure at the exit of the thermal-conductivity detector.

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Details of the physical properties of the solvents used are given in Table I.

TABLE I

PHYSICAL PROPERTIES OF THE SOLVENTS

Solvent	*Molecular Weight*	*Density*	*Coefficient of expansion*	*Reference*
Glycerol	92.06	1.2656 (15°)	0.00055	*a*
Dibutyl phthalate (DBP)	278.3	1.0465 (16°)	0.00058	*b*
Dinonyl phthalate (DNP)	248.3	0.9537 (16°)	0.00056	*b*
Silicone 702	530	1.07 (25°)	0.00086	*c*
Water	18.0	1.000 (4°)	—	*d*

[a] *International Critical Tables.*

[b] Density and coefficient of expansion measured experimentally.

[c] Data sheet on Silicone 702, Edwards and Co., London.

[d] The volume of water at the column temperature was calculated from the density at that temperature (from reference [a]).

The halogenated hydrocarbons were of A.R. quality wherever possible but any small amounts of impurities were separated in the gas–liquid column and did not affect the experimental retention volume. Fluoro-hydrocarbons were supplied by I.C.I. Ltd., and by the Imperial Smelting Corporation Ltd., and samples of separated *cis*- and *trans*-dichloroethylene by I.C.I. Ltd.

Vapour pressures, latent heats of vaporisation, and critical point data were taken from *International Critical Tables*, TIMMERMANS[13], and MAXWELL[14]. Antoine equations calculated from DREISBACH's data[15] were used to derive vapour pressures for those solutes for which direct vapour pressure–temperature data were not available. Fugacity coefficients were calculated from the reduced critical point data and fugacity coefficient charts[14, 16].

Method for the calculation of partition coefficients and activity coefficients

(i) The retention time was measured for 1 μl (or less than 1 μl) of liquid solutes and for 0.1 ml N.T.P. of gaseous solutes. These amounts had previously[13] been found to give retention volumes which were within 1% of the "limiting retention volume" for zero sample size, as determined by the elution of a series of samples of varying size. The sample size is indicated in the following Tables where retention data were available only for liquid samples of > 1 μl (or > 0.1 ml of gases).

(ii) The "corrected retention volume", V_R°, was obtained by multiplying the retention time (corrected for the "dead-time" of the apparatus) by the "corrected flow-rate". The latter was obtained by correcting the measured flow-rate (a) for the vapour pressure of water at the flow-meter temperature, (b) for the pressure drop across the column (by the formula given by JAMES AND MARTIN[17]) and (c) to the temperature of the column.

(iii) The partition coefficient, k, was obtained by dividing V_R° by the volume of the stationary liquid phase at the column temperature.

(iv) The activity coefficient at infinite dilution, γ°, was obtained in the form of γ_p° or γ_f° from the formulae:

$$\gamma_p^\circ = \frac{N_{\text{liq.}}RT}{k.p^\circ} \quad \text{or} \quad \gamma_f^\circ = \frac{N_{\text{liq.}}RT}{k.f^\circ}$$

where p° is the vapour pressure, and f° is the fugacity, of the solute at temperature T, and $N_{\text{liq.}}$ is the number of moles of liquid phase per unit volume at temperature T.

RESULTS

The reproducibility of γ_p° for retention times measured at approximately the same temperature but with varying operating conditions during routine analyses is shown in Table II.

TABLE II

THE REPRODUCIBILITY OF k AND γ_p° FOR VARYING CONDITIONS

Liquid phase	*Weight of liquid phase, g*	*Column temperature, °C*	p_i/p_o	*Solute*	*Volume of solute, μl or ml*	k	γ_p°
Dinonyl phthalate	1.89	57.6	1.275	CH_2Cl_2	1 μl	98	0.43
	1.86	56.2	1.490		1 μl	94	0.46
	1.89	57.2	1.182		0.5 μl	92	0.47
	1.89	57.6	1.275	$CHCl_3$	1 μl	234	0.35
	1.86	56.2	1.490		1 μl	227	0.37
	1.89	57.2	1.182		1 μl	230	0.36
	1.89	19.1	1.172	$CFCl_3$	0.1 ml	99	0.78
	1.89	20.3	1.077		0.1 ml	98	0.76
	1.89	19.4	1.129		0.1 ml	104	0.74
Silicone 702	1.25	57.0	1.382	CCl_4	1 μl	214	0.48
	2.14	55.7	1.246		1 μl	204	0.51
	2.14	55.7	1.120		1 μl	203	0.51
	2.14	55.7	1.246	CH_2Br_2	1 μl	392	0.58
	2.14	57.6	1.316		0.1 μl	385	0.59
	2.14	55.7	1.120		1 μl	397	0.57

The mean values of k and γ_p° for a number of halogenated hydrocarbons at a series of temperatures are given for DNP, Silicone 702, and glycerol in Tables III, IV and V respectively. Values of k and γ_p° for three chloromethanes and methanol in water are given in Table VI and for a few hydrocarbons and oxygenated compounds in DBP, DNP, and Silicone 702 in Table VII.

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TABLE III

VALUES OF k AND γ_p° FOR HALOGENATED HYDROCARBONS IN DINONYL PHTHALATE

References to sample size, (*a*) 0.3 ml N.T.P., (*b*) 5 μl, (*c*) 10 μl, (*d*) 0.4 ml N.T.P.

Temperature, °C	*19.3*		*23.6*		*24.5*		*39.7*		*57.4*		*76.8*		*97.9*	
Solute	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°
CH_3Cl			27.3	0.45	27.0	0.44					9.15[a]	0.45		
CH_2Cl_2	331[b]	0.44					170.5	0.42	98.2	0.43	55.7	0.46	32.4	0.49
$CHCl_3$							451	0.33	232	0.35	122.4	0.38	71.5	0.37
CCl_4							353	0.71	192.4	0.70	107.2	0.71	62.2	0.71
CH_2ClBr											120.5	0.49	71.0	0.48
CH_2Br_2											250	0.60	133.5	0.61
$CHBr_3$													600	0.64
cis-Dichloroethylene							366	0.38			107.7	0.42	64.5	0.42
trans-Dichloroethylene							173.5	0.52			57.8	0.58	32.6	0.60
Trichloroethylene							767[c]	0.46			187.8	0.55	100.5	0.60
Tetrachloroethylene											390	0.80	202	0.79
Ethylene dichloride											164.5	0.57		
CF_3H					1.84	0.82								
CF_3Cl					0.58	3.4								
CF_2Cl_2	10.0	1.16	9.3	1.12	9.39	0.91					3.78[d]	0.80		
$CFCl_3$	104.2	0.74	89.0	0.75	80.6	0.80					23.4	0.66		
$CFCl_2H$			139.5	0.28	130.5	0.29								
CF_2ClH			18.1	0.58	18.0	0.57								

TABLE IV

VALUES OF k AND γ_p° FOR HALOGENATED HYDROCARBONS IN SILICONE 702

References to sample size, (*a*) 0.3 ml N.T.P., (*b*) 0.2 ml N.T.P., (*c*) 2 μl.

Temperature, °C	*20.2*		*21.9*		*23.1*		*40.1*		*55.7*		*77.0*		*97.9*	
Solute	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°	k	γ_p°
CH_3Cl					23.1	0.36					8.07[a]	0.37		
CH_2Cl_2	278	0.38					128.1	0.40	74.0	0.44	42.6	0.44	22.4	0.50
$CHCl_3$	710	0.32					288	0.37			85.8	0.40	42.4	0.45
CCl_4	891	0.45					363	0.50	203.5	0.51	109.4	0.50	55.3	0.58
CH_2ClBr	730	0.42					306	0.45	162	0.47	91.1	0.47	48.5	0.51
CH_2Br_2							797	0.52	360.5	0.59	185.0	0.59	95.8	0.61
$CHBr_3$											915	0.65		
cis-Dichloroethylene											79.0	0.42	38.6	0.51
trans-Dichloroethylene											50.0	0.46	26.6	0.53
Trichloroethylene													76.3	0.57
CH_3I	238[c]	0.46												
CHF_3							0.53[b]	1.61						
CF_3Cl			0.84	1.83			0.63	1.80						
CF_2Cl_2			10.8	0.75	10.0	0.78	6.90	0.76			4.12	0.63		
$CFCl_3$			93.1	0.56	88.8	0.56	55.0	0.53			21.8	0.52		
$CHFCl_2$					80.9	0.36	47.8	0.37			19.4[b]	0.38		
CHF_2Cl			10.4	0.79	10.4	0.76					3.76[a]	0.71		

TABLE V

VALUES OF k AND γ_p° FOR HALOGENATED HYDROCARBONS IN GLYCEROL

Temperature, °C	*25.6*		*28.2*		*40.1*		*77.0*		
Solute	*k*	γ_p°	*k*	γ_p°	*k*	γ_p°	*k*	γ_p°	ln γ°
CH_3Cl			2.82	20.1	2.67	14.8			
CH_2Cl_2	17.2	33.8			13.5	25.7	7.13	17.8	2.88
$CHCl_3$	21.1	59.6			18.3	39.2	8.65	27.2	3.30
CCl_4	5.45	415			5.4	226	3.5	109	4.69
CH_2ClBr			48.3	31.7	31.6	29.8			
CH_2Br_2					72.3	38.7	25.4	29.7	3.39
$CHBr_3$							91.8	44.6	3.80
cis-Dichloroethylene					15.9	43.9	7.40	31.0	
trans-Dichloroethylene					5.9	76.7	3.87	40.6	
Trichloroethylene							6.74	76.0	4.33
Tetrachloroethylene							5.7	274	5.61
Ethylene dibromide							37.5	59.4	4.08

TABLE VI

VALUES OF k AND γ_p° FOR CHLORO-METHANES AND METHANOL IN WATER

Solute (1 μl)	*Wt. of H_2O, g**	*Temperature °C*	V_R° *ml*	*k*	γ_p°
CH_2Cl_2	1.57	23.4	30.0	19.1	140
	1.12	24.1	15.1	13.6	180
	0.48	27.3	2.85	5.94	370
$CHCl_3$	1.56	23.4	19.4	12.5	310
	1.05	24.1	10.4	9.94	540
	0.38	27.3	3.15	8.20	580
CCl_4	1.59	23.4	3.69	2.31	4150
	1.07	24.1	3.03	2.83	3300
	0.45	27.3	2.76	6.14	1300
MeOH	0.41	27.3	1380	3360	2.2

* Weight in g on 3 g kieselguhr (corrected for loss of water to gas phase as described previously[13]).

TABLE VII

VALUES OF k AND γ_p° FOR VARIOUS SOLUTE–SOLVENT SYSTEMS

Solvent	*Temperature °C*	*Solute*	*Amount of solute μl*	*k*	γ_p°
DNP	57.2	H_2O	< 0.01	51	8.27
		H_2O	1	28	15.0
DNP	56.5	MeOH	2	39.9	2.55
		EtOH	2	85.5	2.14
		iso-PrOH	2	120.6	1.83
DNP	57.6	Acetone	< 0.01	69.3	0.99
	57.2	Acetone	2.5	62.5	1.13
DNP	39.5	Acetone	2.5	114.8	1.10
DBP	17.7	MeOH	< 0.01	268	2.99
		EtOH	< 0.01	571	3.14
Silicone 702	16.8	Isopentane	0.5	78.9	0.92
		n-Pentane	1.5	112.1	0.88
		2-Me-pentane	< 0.01	241	1.02
		3-Me-pentane		288	0.96
		n-Hexane		368.6	0.95
		2,4-Di-Me-pentane	1	546.3	1.00

DISCUSSION

Comparison of γ_p° values for halogenated hydrocarbons with those for other substances in polar and non-polar solvents

For the solvents DNP and Silicone 702, γ_p° values for halogenated hydrocarbons are < 1, with only three exceptions, *viz*: $\gamma_p^\circ = 1.1–1.2$ for CF_2Cl_2 at 20°, $\gamma_p^\circ = 1.6–1.8$ for CF_3Cl and CF_3H at 20–30°. However, the last two substances gave very low retention volumes, which were therefore difficult to measure accurately. CF_2Cl_2 gave the value $\gamma_p^\circ = 0.8$ at 77°, and from the limited data available appears to have $\gamma_p^\circ < 1$ at temperatures $> 40°$. With glycerol as the solvent all the halogenated hydrocarbons gave $\gamma_p^\circ \gg 1$. The few results with chloromethanes in H_2O as solvent gave $\gamma_p^\circ > 100$ and the values depended upon the amount of solvent.

In general therefore for halogenated hydrocarbons γ_p° is < 1 in non-polar or relatively non-polar solvents, and $\gg 1$ in highly polar solvents. These results are very similar to those found for a wide range of hydrocarbons in polar and non-polar solvents, *e.g.* in non-polar hydrocarbon solvents γ° is < 1, in the relatively non-polar di-isodecyl phthalate γ° is $<$ or $\simeq 1$, and in highly polar polyglycol solvents aliphatic hydrocarbons have large values of γ° (17–1.3) and aromatic hydrocarbons have values ranging from 2.3–0.5.

The relationship of γ° to the transport of solutes through gas–liquid columns

Values of $\gamma^\circ < 1$ are found if the components of the solution are similar in polarity but have a large difference in molecular magnitude, or if the components tend to form transient adducts[4], and "negative" deviation from Raoult's law usually results. The value of k is lower for a concentrated part of a zone of solute than for a dilute part. Hence the leading edge of a zone is sharpened as it travels through a column, and the tail is dispersed. The retention volume of the maximum of a relatively concentrated zone is therefore less than that of an infinitely dilute zone. A graphical example of this effect has previously been given[12] for a zone of $CHCl_3$ eluted through a column containing DNP. This effect applies for all the above halogenated hydrocarbons in DNP and in Silicone 702.

Values of $\gamma^\circ > 1$ are found if there is a large difference in the polarity of the solute and the solvent. The value of k is greater in the more concentrated part of a zone than in dilute parts. The elution curve has a sharpened tailing edge and a diffuse front, and the retention volume of the zone maximum is greater than in the limiting case of "zero" concentration.

The above effects are most important in the first part of a column where the concentration of a solute is highest, and they become smaller as the column length is increased and the sample size is reduced. Hence for the measurement of accurate values of γ°, small samples and long columns (3–6 m) are recommended[4].

Variation of γ° with temperature

The variation of γ_p° with temperature for halogenated hydrocarbons depends upon the particular hydrocarbon and upon the solvent. Values of γ_p° in glycerol decrease

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with increase in temperature. With DNP, γ_p° increases with temperature for CH_2Cl_2, trichloroethylene, and *trans*-dichloroethylene, remains approximately constant for CCl_4, CH_2Br_2, CH_2ClBr, CH_3Cl, $CHCl_3$, *cis*-dichloroethylene, and tetrachloroethylene, and decreases with increase in T for CF_2Cl_2 and $CFCl_3$. With Silicone 702, γ_p° increases with T for CH_2Br_2, CH_2Cl_2, $CHCl_3$, CCl_4 and CH_2ClBr, remains constant for CH_3Cl, $CFCl_2H$ and $CFCl_3$, and decreases for CF_2Cl_2 and CF_2ClH.

KEULEMANS[4] and KWANTES AND RIJNDERS[5] find that γ_f° varies very little with T, and that γ_p° varies to a greater extent, as would be expected since γ_p° is a measure of the combined imperfections in the gas and liquid phases. However, for the majority of the halogenated hydrocarbons under the conditions studied the calculated fugacity coefficients are close to unity and therefore $\gamma_p^\circ \simeq \gamma_f^\circ$. For several solutes γ° shows a large variation with T, and since this effect is not in agreement with results for non-halogenated hydrocarbons a more accurate determination of γ° as a function of T would be desirable.

It is of interest to note the difference between the temperature-variation of k, and of γ°. For all the substances studied k is approximately linearly dependent on $1/T$, where T is the absolute temperature, whereas γ° shows the above-mentioned complex dependence on T. The tabulation of retention data as plots of k against $1/T$ or $\log V_g$ against $1/T$ is therefore more convenient than plots of γ° as a function of T. Considerably more experimental data of higher accuracy than have already been reported will be required before the temperature dependence of γ° can be satisfactorily explained.

The accurate determination of γ° by gas–liquid chromatography

The requirements for the accurate evaluation of γ° from measurements with gas–liquid columns can be summarised as follows:

(a) Very small sample size, or extrapolation to zero size from a range of small samples (the latter is recommended).

(b) Long columns (explained previously).

(c) Accurate measurement of retention time, dead-volume of apparatus, temperature and pressure of flow-meter, inlet and outlet pressures at the column, and column temperature.

(d) Accurate determination of the mass of the solvent and, if k is also required, the volume of the solvent at each column temperature, which requires a knowledge of the coefficient of expansion of the solvent.

(e) The molecular weight of the solvent, which implies that γ° values cannot be accurately evaluated for solvents which contain a mixture of substances in unknown proportion (unless they are isomers) or for polymers for which accurate molecular weights cannot be determined.

For the values of γ° to be fundamentally sound they should be independent of specific parameters that determine the column efficiency. Whereas this is usually the case[5,8] (provided that the amount of solvent on an adsorptive support such as Celite is at least 15% by weight[5]) it should be confirmed for the particular solute–solvent combination for which γ° is to be determined. With chloromethanes in water on Celite the values of k and γ° are not independent of the amount of water and further work is required to elucidate the interactions in this system. The use of a non-polar support, *e.g.* fine metal helices, is recommended[5], particularly for polar solutes in non-polar solvents. The basic method can be extended to solvents of relatively high volatility by presaturating the carrier-gas with the vapour of the solvent, and the value of γ° for a *n*-hydrocarbon in its next higher homologue can then be determined.

Highly accurate values of γ° can be evaluated if the above requirements are satisfied. Gas–liquid chromatography will then be applicable to the detailed study of the thermodynamics of volatile solutes in volatile and involatile solvents, for which it is difficult to derive γ° by the classical vapour-pressure measurements.

SUMMARY

Activity coefficients at infinite dilution, γ°, have been calculated for a series of halogenated hydrocarbons in dinonyl phthalate, glycerol, and Silicone fluid 702 over the range 20–100°. Values of γ° for several aliphatic hydrocarbons and oxygenated compounds have also been calculated.

The values of γ° for halogenated hydrocarbons are compared with those for other substances in polar and non-polar solvents and are discussed in relation to the transport of solutes through gas–liquid columns. Considerable deviations from ideality have been found for halogenated hydrocarbons in highly polar solvents, *e.g.* for halogenated methanes at 25° $\gamma^0 > 10$ in glycerol and > 100 in water. The variation of γ° with temperature has been shown to depend upon the particular solute and upon the solvent.

A summary is given of the experimental requirements for the accurate determination of γ° by gas–liquid chromatography.

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A QUANTITATIVE STUDY OF THE RELATION BETWEEN MOLECULAR STRUCTURE AND ADSORPTION AFFINITY ON SILICIC ACID-CELITE COLUMNS

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INTRODUCTION

The field of partition chromatography, especially as applied to gases, has moved ahead with great strides in recent years. A good deal of this advance was brought on by a correlation by MARTIN[1] of structure and development rate in partition chromatography. On the other hand, adsorption chromatography has shown no such advance; among the reasons for this is that no similar correlation has been successfully developed in this area. In the present undertaking a step in this direction has been attempted.

There have been innumerable studies of the relation between the structure of a molecule and its adsorption affinity during chromatographic adsorption analysis, but comparatively few of these have been quantitative[2, 3, 4]. CLAESSON[2] and HALL AND TISELIUS[3] worked with essentially homologous series of aliphatic acids and esters adsorbed on charcoal and thus were not concerned with a broad variety of structural types. LEROSEN and his co-workers[4] made the first concerted and systematic effort to relate the structure of a molecule to its adsorption affinity on silicic acid. Unfortunately, however, the interpretation of much of this ambitious work is ambiguous for a number of reasons[5]: first, the conditions of chromatography were such that both partition and adsorption effects[6] were probably operating because they used unheated (inactive) silicic acid instead of heated (activated) material for most of their work. Consequently many of the effects observed probably result at least as much from partition between the mobile phase and an immobile, primarily aqueous, phase as from ordinary adsorption. A second feature of the work which could lead to ambiguity was the use of 0.01 M sample solutions of all compounds studied; during the elution analysis technique which they employed the concentration of the solute will decrease as the zone moves down the column, and if the isotherm is non-linear the development rate will thus change as well. It has been found in the present work that for some compounds development rates vary with concentration even down to concentrations of 10^{-4} M. Finally, the 12-parameter (and later 6-parameter) equations proposed for

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correlation of the data were entirely empirical and the parameters themselves were at best rather poorly defined.

In the present investigation silicic acid (finely powdered silica gel) was chosen as the adsorbent because it has been shown to be very satisfactory for chromatographic purposes[7], because adsorption isotherms on silicic acid are frequently linear or nearly so[8], and because of the role that hydrogen bonds play in adsorption on this and similar adsorbents. Heated adsorbent and anhydrous solvents were used in order to ensure that the processes studied involved adsorption effects only.

Benzene was selected as the solvent for all the work presented here because of its ready availability in pure form, its non-polar character and its good solvent properties for and similarity to the aromatic compounds used in this work. The interpretations of the adsorption isotherms of the compounds studied were attempted only in the linear region of the isotherm to avoid the additional complications which arise in an attempt to correlate the structure of compounds with the entire isotherm. The development rates were calculated for infinite dilution of the solute which is necessarily in the linear region of the isotherm. Quantitative correlations of the data were sought through the utilization of thermodynamic relationships similar to those developed for partition chromatography[1, 9–11].

THEORETICAL

The relative adsorption affinity of a compound is defined here as the equilibrium constant corresponding to the standard state free energy change for the transfer of one mole of solute, S, from solution to the adsorbed state. The process to be considered may be formulated as:

$$S_{solution} = S_{adsorbed} \tag{1}$$

The standard free energy change may be written,

$$\Delta F^\circ = -R_gT\ln(S_a)/(S_s) = -R_gT\ln K \tag{2}$$

where the gas constant is given the subscript "g" to distinguish it from the development rate, R. The subscripts s and a refer to the solution and adsorbed states respectively and the terms in parenthesis represent concentrations. K is therefore the equilibrium constant for this process, and will be termed the adsorption affinity of the solute.

A similar equilibrium is established by the developer

$$D_s = D_a \tag{3}$$

The equilibria represented by reactions (1) and (3) may be independent of each other or may influence one another. If they are independent of one another then, for a series of solutes chromatographed in the same solvent, reaction (3) need not be considered further. However, if they are interrelated the overall reaction for one mole of solute may be written,

$$S_s + mD_a = S_a + mD_s \tag{4}$$

The standard free energy change then is

$$\Delta F^{\circ} = -R_gT \ln (S_a)(D_s)^m/(S_s)(D_a)^m = -R_gT \ln (S_a)/(S_s) - R_gT \ln (1/K')^m = \\ = -R_gT \ln K + \text{constant} \tag{5}$$

where $K' = (D_a)/(D_s)$ and is approximately constant when dilute solutions of solutes are studied*. The terms (S_a) and (S_s) in equations (2) and (5) are proportional respectively to $f(c)$ and c of the familiar Langmuir isotherm:

$$f(c) = ac/(1 + bc) \tag{6}$$

For the present purposes it is not necessary to know the exact proportionality constants between them because in equations (2) and (5) only the dimensionless ratio $(S_s)/(S_a)$ is required; therefore, it is only necessary to express $f(c)$ and c in identical units. If c is in moles per liter and $f(c)$ in moles per gram of adsorbent then $f(c)/c$ must be multiplied by M/α in order to make it dimensionless. M is the average weight of adsorbent packed in the column per unit of column length and α is the interstitial volume per unit length of packed column[12].

Thus,

$$(S_a)/(S_s) = (M/a)f(c)/c = K \tag{7}$$

and equation (2) becomes

$$\Delta F^{\circ} = -R_gT \ln (M/a)f(c)/c \tag{8}$$

Equation (5) is similarly altered.

In chromatography the experimentally measurable quantities are the distances that the zone of solute and the front of solvent have travelled in the same period of time. Their ratio, familiarly designated R, is the development rate of the solute; it represents the fraction of time the solute remains in solution during the chromatogram[13]. It has been shown[13] that

$$(1 - R)/R = (M/a)f(c)/c \tag{9}$$

and thus equation (8) becomes

$$\Delta F^{\circ} = -R_gT \ln (1 - R)/R \tag{10}$$

When the concentration, c, is so low that the bc term in equation (6) becomes insignificant with respect to unity the isotherm becomes linear and then

$$(1 - R^{\circ})/R^{\circ} = (M/a)f(c)/c = (M/a)a = K \tag{11}$$

where R° represents the development rate in the linear region of the isotherm. Sometimes the concentration at which the development rate becomes constant is so low as to be difficult to detect by ordinary means; it is then necessary to obtain R° by an appropriate extrapolation. By combining and rearranging equations (6), (7) and (9), one can obtain two equations which are closely related to each other:

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* In the linear region of the isotherm, which corresponds at least to the dilute solution region of the solute, the term $(S_a)/(S_s)$ is constant; consequently if the entire quotient is constant, $(D_a)/(D_s)$ must also be constant.

$$R/(1-R) = (b/K)c + 1/K \tag{12}$$

$$(1/c)R/(1-R) = 1/Q = (1/K)1/c + b/K \tag{13}$$

From (12) a plot of $R/(1-R)$ against c has slope b/K and intercept $1/K$; from (13) a plot of $1/Q$, *i.e.*, $R/c(1-R)$ against $1/c$ gives slope $1/K$ and intercept b/K. If the data fit the Langmuir isotherm perfectly, then of course one obtains the same b and K from either equation; in practice errors in the data as well as possible departures from the Langmuir equation may cause slight variations. Although (12) might in principle be considered better because it emphasizes more the data at low concentration, these data are likely to be less accurate because of difficulties in locating zones precisely at low concentration. As discussed later, we found in practice that (13) gave more consistent results, although the differences were small. The value of ΔF° for any solute on columns of silicic acid in the linear region of the isotherm is thus

$$\Delta F^\circ = -R_gT \ln K = -R_gT \ln (1-R^\circ)/R^\circ \tag{14}$$

The standard free energy change defined by the above equation corresponds to the adsorption process described in reaction (1).

In the present paper it is postulated that this standard state free energy change is, to a first approximation, expressible as the sum of the standard free energy changes for the component groups of atoms which comprise the molecule. For the compounds studied here, one of these groups will be the aromatic nucleus and the others will be the various substituent functional groups, all of which here are capable of forming rather strong hydrogen bonds. The standard state free energy change is therefore expressed as

$$\Delta F^\circ = \sum_i \Delta F_i^\circ \tag{15}$$

where the subscript i designates the individual component groups of the molecule. It is understood that certain additional correction terms will often be required in equation (15), especially for polyfunctional compounds. In particular, a geometrical correction term* may sometimes be needed when different adsorbing groups in the molecule bear such a steric relation to one another that their simultaneous attachment to adsorption sites is hindered, and what might be called an electronic correction term may be needed on occasion when there are marked changes in the hydrogen-bonding affinity of a group because of very marked resonance or inductive interactions with other substituents. Still another term might be needed to correct for possible changes in interfacial tension between adsorbent and solvent upon adsorption. As a first approximation, however, these correction terms have been assumed for the present studies to be negligible.

Equation (15) implies that

$$K = \prod_i K_i \tag{16}$$

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* This geometrical term would be extremely important when adsorbents are specially prepared to "fit" a specific molecule (for example, silicic acid precipitated in the presence of methyl orange as described by DICKEY[14]).

i.e., that the overall adsorption affinity as here defined is the product of the individual group adsorption affinities.

EXPERIMENTAL

Materials

The reagents used were of reagent grade or better; the chromatographic equipment was standard. The adsorbent consisted of a mixture of silicic acid (Mallinckrodt reagent grade, code number ZSA) with the essentially inert filter-aid Celite (Johns Manville No. 503, washed with diethyl ether and dried) in a 1.75:1 ratio by weight of the anhydrous powders. The adsorbent mixture was activated at 135° for a few hours before use.

Chromatographic procedures

Columns were packed in a manner similar to that described previously[8] except that great care was taken to avoid absorption of moisture by the adsorbent. The only driving force for solvent flow during chromatography was gravity; no reduction in pressure was used. Precautions were taken to ensure that the chromatographic solution was in equilibrium at all times with the adsorbent. The chromatograms were all run by the frontal analysis technique[2] for a variety of practical as well as theoretical reasons. Zone fronts of the various solutes were usually determined by carefully extruding the column onto a sheet of paper and streaking with suitable streak reagents along different sections and cross-sections of the column (seven sections in all, including the longitudinal axis). A weighted average of these values was used in determining the development rate. The streak reagents used to detect most of the compounds studied in this investigation have been used previously for similar compounds[15-19], although many slight modifications[5] had to be made to improve their effectiveness. Only two compounds, nitrobenzene and *p*-nitrobiphenyl, could not be detected by the streak reagent technique; the streak reagents recommended[15, 16] for these substances proved unsuccessful. Zone fronts of these two compounds were detected by collecting 0.08-ml fractions flowing out of the column and determining their absorbance at 320 mμ (near the absorption peak for each compound). The lowest limit of concentration detected was 3.6×10^{-3} M for nitrobenzene and 10^{-4} M for *p*-nitrobiphenyl.

Column constants

V_{150} values and interstitial volumes, α, were determined in tubes packed in the standard manner[6, 7, 12]. The interstitial volume of a 150-mm column does not remain constant but rather increases by about 2% every 20 mm within the first 60 mm and then becomes essentially constant below about 80 mm. These values are reproducible to about 1.5%. In order to make development rates comparable, the V_{150} value was chosen which corresponded to the distance along the column subsequently to be occupied by the zone.

To determine the linear density, M, along the column a packed column was carefully extruded and divided into 5-mm sections in an apparatus designed for this purpose[20]. The linear packing density is 8% higher in the top 5 mm of the column (0.0271 g/mm) than in the next 40 mm (0.0250 ± 0.0005 g/mm); in the next 20 mm, the density is once again identical to the density in the first five millimeters (0.0271 ± 0.0004 g/mm). The increased packing density at the top of the column has been noted previously[6] and probably occurs during the smoothing of the upper surface after packing the column. However, with columns to which a differential pressure of nearly one atmosphere is applied during chromatography the density distribution in the remainder of the column is somewhat different[6] and the average density is 10–15% higher than in the present columns.

R° calculations

In the present investigation the frontal analysis method[2] was chosen for the determination of R values. Consequently the concentration of solute in the interstices of the column (required in equations (12) and (13)) was known and was equal to the concentration of solute in the solution poured on the column. To minimize systematic errors during the determination of $\overline{X}$, the average distance travelled by the zone front, and l, the distance that the solvent progressed in the same time, a differential method was used for the determination of development rates. The equation used was

$$R = (\overline{X}_2 - \overline{X}_1)/(l_2 - l_1)$$

where the subscripts refer to determinations of $\overline{X}$ at different values of l. In the determination of R° for a compound, four identical chromatographic runs were made at each of three different concentrations in benzene. The concentrations used varied by a factor of nine on the average, with a minimum variation by a factor of two (benzyl alcohol) and a maximum variation by a factor of one hundred (*p*-nitrobiphenyl). No concentration was greater than $10^{-2}\,M$; the average concentration was between 10^{-3} and $10^{-4}\,M$ and the lowest concentration was about $10^{-5}\,M$. The lower limit of concentration used for each compound was governed by the lowest concentration at which the zone of the compound was detectable with the streak reagent used[5].

With three values for the independent variable c in equation (12) or $1/c$ in equation (13), and four values for the corresponding dependent variable at each concentration, there were twelve items for the evaluation of the constants K and b in equations (12) and (13) by the method of least squares. In the evaluation by (13), a weighting factor was used; each observational equation was multiplied by a factor proportional to $c^{0.5}$ because the $1/c$ term in $1/Q$ tends to magnify the variance in the $R/(1 - R)$ term in (13) to an extent estimated to be approximately proportional to the magnitude of $1/c$. The data were processed on the National Bureau of Standards Western Automatic Computer (SWAC).

Equilibrium

For the classical thermodynamic treatment used in the present study to be valid the system must be at equilibrium. The equilibrium between solute adsorbed and solute in solution during chromatography is disturbed by the flow of solution past the adsorbent particles. In all the present chromatographic experiments we used gravity flow, about 0.10 to 0.20 ml/min in 9-mm (i.d.) columns. A study of the extent to which equilibrium is established with these flow rates was made with solutions of *p*-nitrodiphenylamine in benzene. As indicated in Table I, the constants in the Langmuir isotherm equation calculated from the observed chromatographic development rates agreed, within the limits of experimental error, with those evaluated from the static adsorption isotherm determined for the system by equilibration of solution and adsorbent for 30 minutes. Although the results calculated from both (12) and (13) agreed with each other and with the static isotherm values within the estimated standard deviations, those obtained from (13) seemed a little more consistent. Consequently, (13) was used for all of the values quoted subsequently.

TABLE I

LANGMUIR CONSTANTS AND CORRESPONDING R° FOR *p*-NITRODIPHENYLAMINE IN BENZENE

Method of evaluation	*a (ml/g)*	*b (M^{-1})*	R°
Chromatographic (equation (12))	9.6 (± 0.6)	$9\ (\pm 1) \times 10^1$	0.165 (± 0.008)
Chromatographic (equation (13))	9.0 (± 0.4)	$6\ (\pm 2) \times 10^1$	0.172 (± 0.006)
Static isotherm	8.9 (± 0.3)	$8\ (\pm 3) \times 10^1$	0.174 (± 0.005)

There was a slightly greater discrepancy for finite concentrations than for the infinitely dilute state. For example, for a $2.34 \times 10^{-3}\ M$ solution in benzene, the average R was 0.187 ± 0.008, whereas a calculation at this concentration from the adsorption isotherm, in which each point was obtained after a 30-minute equilibration of the solution with the adsorbent, gave $R = 0.201$, about 6% *larger* than that evaluated chromatographically. Since the direction of this discrepancy is opposite to that expected if the rate of attainment of equilibrium were important, it seems more probable that the discrepancy represents the effect of experimental errors in the two different methods of obtaining R-values. The R-value calculated from the static adsorption isotherm was based on the average (M/α) value determined for those columns with which the experimentally determined R-values were compared. It is possible that a 6% variation of this average (M/α) value from the actual (M/α) value in the column in with the R-value was experimentally determined might be the sole cause of the discrepancy.

In a series of experiments in which R was determined as a function of flow rate, it was found that a plot of R against the logarithm of the flow rate indicated that a limiting value of R was reached at very low flow rates, and this limiting value was within 1% of the R-value determined at gravity flow-rates. Similarly a plot of R against the square root of the flow rate[21] yields a straight line whose intercept at zero flow rate gives a value of R which is within 5% of the gravity flow value.

The foregoing evidence indicates that equilibrium does exist on silicic acid under the conditions used throughout the present investigation, as has been found before[8].

Errors

A detailed consideration[5] of possible sources of errors arising from variation in column packing (M/α), and from inaccuracies in determinations of concentration, solvent position, and zone position, indicated that errors from the first of these sources were in most cases by far the most important. However, with strongly adsorbed compounds, the distance of travel of the zone front was so small that errors in measurement of its position contributed significantly to the overall error (*e.g.*, with benzylamine). The errors quoted in this paper are standard deviations evaluated in the usual way from the least squares equations.

RESULTS AND DISCUSSION

It has for some time been recognized that hydrogen bonding plays a key role in the adsorption process on silicic acid[22-26]. Since decreasing the number of hydroxyl groups present on silicic acid by heating it at elevated temperatures (above 150°) decreases its adsorption affinity[26-28] it may be concluded that the adsorption sites are the hydroxyl groups[29] and that these very weakly acidic hydroxyl groups hold the solute by hydrogen-bond formation both from the adsorbent's hydroxyl groups to the solute and from an appropriate hydrogen on an electronegative atom of the solute to the oxygen of the hydroxyl group. The evidence of discrete sites on silicic acid permits the description of the adsorption process to be made with the use of stoichiometric equations and justifies the use of the Langmuir equation (6) in the calculation of adsorption affinities in the linear region of the isotherm.

Studies on the nature of silicic acid particles[30] reveal that they consist of aggregates of primary particles[31] made up essentially of a three-dimensional network of silicon-oxygen tetrahedra with occasional acidic hydroxyl groups in place of oxygen atoms[32]. The arrangement of the atoms varies from a cristobalite structure[33] in certain microcrystalline regions to a linear or branched-chain random arrangement[30, 32]. On the average a distorted cristobalite structure exists with two acidic hydroxyl units for each surface silicon atom and eight such hydroxyl units per square millimicron of surface (this corresponds to about 5% "structural" water, which is about what is found in commercial samples of silicic acid). The distance between the centers of the oxygen atoms of hydroxyl groups attached to the same silicon atom may be calculated to be about 2.6 Å on the assumption of a tetrahedral angle at the silicon atom and an Si–O distance of 1.6 Å[34]. The distance between oxygens of hydroxyl groups on adjacent silicon atoms may be calculated (with the above assumptions and an assumed[34] Si–O–Si angle of 150°) to be between 4.3 Å and 5.8 Å depending upon the situation of the groups. These distances are of the order of magnitude of the distances between the adsorbing atoms on the *meta* and *para* position isomers.

It is possible that the distance between adsorbing atoms which is most favorable for maximum adsorption on silicic acid might be revealed by a comparison of the interadsorbing atom distances in pairs of isomers with the adsorption affinities of these isomers. This comparison was made for the compounds shown in Table II and the evidence suggests that the most favorable interadsorbing atom distance is about 6.1–6.2 Å or some multiple thereof which is close to the 5.8 Å value calculated above. Although it is evident from Table II that the isomer whose interadsorbing atom distance is closest to the favorable 6.1–6.2 Å is the more strongly adsorbed, it would appear that if geometric factors alone are responsible for the observed effects, the difference in the geometric factors between the *meta* and *para* isomers for the methoxybenzaldehydes should be of the same order of magnitude as for the hydroxybenzaldehydes and the nitroanilines. A check of the ($\Delta F^\circ_m - \Delta F^\circ_p$) values or the log ($Km/Kp$) values in Table II for these compounds reveals that this is not the case. On the other hand, the differences in the standard free energy changes for the isomeric compounds are so small that it might even be thought surprising that consideration of interadsorbing atom distances alone yields even qualitative information concerning the relative order of adsorption of isomers.

TABLE II

DISTANCES BETWEEN ADSORBED ATOMS IN SOME MOLECULES*

Compound	*Adsorbed atoms*	*Internuclear distance (Å)*		*Adsorption order*	*log K_m/K_p*	*$\Delta F^\circ_m - \Delta F^\circ_p$ (kcal/mole)*
		meta	*para*			
Dimethoxybenzene	O····O	4.8	5.6	$p > m$	—0.025	+0.03
Dinitrobenzene	O····O	4.7–6.8	6.7	$m > p$	0.086	—0.12
Dihydroxybenzene	O····O	4.8	5.6	$p > m$	—0.067	+0.09
Hydroxybenzaldehyde	O····O	5.0–5.9	6.2	$p > m$	—0.19	+0.26
Methoxybenzaldehyde	O····O	5.0–5.9	6.2	$p > m$	—0.33	+0.45
Nitroaniline	N····O	4.9–5.9	6.1	$p > m$	—0.083	+0.11
Nitrobenzaldehyde	O····O	4.8–6.8	6.7	$m > p$	0.22	—0.30

* The distances were calculated from bond distances and angles reported by WELLS[34]. They represent the distances between the principally adsorbed atoms of the molecule.

Table III lists the adsorption affinities (equilibrium constants) and related quantities for adsorption from benzene solutions of the indicated compounds on silicic acid. From this table the relative order of affinities of adsorbing groups for silicic acid may be obtained. In decreasing order of adsorption affinities the sequence appears to be:

$$-CH_2NH_2,\ -COOH,\ -CH_2OH,\ -NH_2,\ CH_3CO-,\ -OH,\ -CHO,\ -NO_2,\ -OCH_3.$$

This order suggests that adsorption occurs mainly by hydrogen bonding from the proton on the silanol group of silicic acid to the solute. This conclusion, in agreement with ILER[29], is drawn from the evidence that the more basic amine is more strongly adsorbed (*i.e.* $-CH_2NH_2 > -NH_2$). That the oxygen atoms of the silicic acid do not play as important a role as acceptors in the adsorption process is clear from the fact that the more acidic hydroxyl group is the less strongly adsorbed (*i.e.* $-CH_2OH > -OH$). However, these silicic acid oxygens apparently do play some role as acceptors in the adsorption process for when the hydrogen atom of the hydroxyl group of a solute is replaced by a methyl group there is an appreciable decrease in the adsorption affinity of the resultant compound (*i.e.* $-CH_2OH > -OH > -OCH_3$). It seems doubtful that this is purely a steric effect of the methyl group interfering with a

TABLE III

ADSORPTION AFFINITIES AND RELATED QUANTITIES* AT 24° ± 1°

Compound	K	σ_K	$R°$	$\sigma_{R°}$	b	σ_b
Acetophenone	15.9	0.2	0.059	0.001	1.6×10^2	0.2×10^2
Aniline	17.6	0.3	0.054	0.001	0.7×10^2	0.5×10^2
Anisole	0.538	0.006	0.651	0.002	-0.1	0.2
Benzaldehyde	5.0	0.2	0.167	0.005	1.8×10	0.8×10
Benzoic acid	82.0	4.0	0.0121	0.006	3.4×10^3	0.1×10^3
Benzylamine	1.6×10^3	0.3×10^3	0.0006	0.0001	3.5×10^3	0.5×10^3
Benzyl alcohol	58.5	3.4	0.017	0.001	2.4×10^2	0.1×10^2
m-Dimethoxybenzene	9.8	0.3	0.093	0.002	12.9×10	0.3×10
p-Dimethoxybenzene	10.4	0.1	0.088	0.001	6.9×10	0.2×10
m-Dinitrobenzene	2.2	0.1	0.309	0.008	3.9×10	0.6×10
p-Dinitrobenzene	1.80	0.03	0.354	0.005	5.3×10	0.6×10
Hydroquinone	189	4	0.0052	0.0001	4.8×10^2	0.2×10^2
m-Hydroxybenzaldehyde	93	2	0.0107	0.0003	0.5×10^2	0.6×10^2
p-Hydroxybenzaldehyde	144	4	0.0069	0.0002	6.6×10^2	0.5×10^2
m-Methoxybenzaldehyde	35	1	0.028	0.001	1.8×10^3	0.2×10^3
p-Methoxybenzaldehyde	75	4	0.0131	0.0008	3.5×10^3	0.3×10^3
p-Methoxybiphenyl	0.53	0.01	0.655	0.006	2.1×10	0.6×10
m-Nitroaniline	18.0	0.6	0.053	0.002	1.0×10^2	0.2×10^2
p-Nitroaniline	21.8	0.2	0.0438	0.0004	9.2×10	0.4×10
m-Nitrobenzaldehyde	15.0	0.4	0.063	0.002	19.3×10	0.8×10
p-Nitrobenzaldehyde	8.9	0.2	0.101	0.002	0.9×10^2	0.2×10^2
Nitrobenzene	0.68	0.02	0.595	0.008	1.9×10	0.6×10
p-Nitrobiphenyl	0.412	0.003	0.708	0.002	0.1×10^2	0.1×10^2
p-Nitrodiphenylamine	4.9	0.2	0.169	0.006	0.8×10^2	0.2×10^2
Phenol	6.0	0.1	0.143	[illegible]	-0.3×10^2	0.2×10^2
Resorcinol	164	8	0.0061	0.[illegible]	5.3×10^2	0.2×10^2
sym-Trimethoxybenzene	33	2	0.030	0[illegible]	[illegible] $\times 10^2$	0.6×10^2
sym-Trinitrobenzene	4.18	0.07	0.193	0[illegible]	[illegible] 10^2	0.6×10^2

* The Langmuir constant, a, is equal to $(a/M)K$; the value of ([illegible]ent work is 1.88 ± 0.02 ml/g. σ_i is the standard deviation of i. The units of b are [illegible]

hydrogen bond to the solute. The fact that the standard enthalpy of adsorption is greater (more negative) by 2.5 kcals per mole for phenol than for an[illegible]ole[5] supports the view that hydrogen bonds *to* the adsorbent play some role.

Except for the position of the amino group in the above sequence, the relative order of affinities is similar to that found by BROCKMANN AND VOLPERS[35] on alumina. Since the hydrogen-bond accepting character of oxygen is greater than that of nitrogen because of the greater electronegativity of oxygen than of nitrogen[36] as well as because oxygen normally has more unshared electron pairs, it might be expected that the adsorption affinity of the amino group would be less than that of the hydroxyl group as it was indeed found to be on alumina. On silicic acid, however, the reverse seems to be true. DOBAY, FU AND BARTELL[37] have found unusually high values of the enthalpy and entropy changes on adsorption of some aliphatic amines on silica gel from which they conclude that a chemical reaction takes place between the amine and the silica gel. The evidence found here also suggests that some reaction other than simple hydrogen bonding occurs with amines. Spectroscopic evidence[38] rules out salt formation by complete proton transfer as a possible reaction to be considered, but does suggest that the weakly acidic character of the silicic acid promotes a greater interaction with aniline (and presumably with other amines) than occurs between water and aniline, although the interaction with the adsorbent is appreciably less than that between aniline and dilute hydrochloric acid.

Quantitative information

Functional group adsorption affinities

In order to obtain the adsorption affinity values of the component adsorbing groups of a molecule a value for the adsorption affinity of the substituted aromatic nucleus had first to be determined. This value was obtained by solution of the appropriate simultaneous equations for the adsorption affinities of the compounds listed in Table III. For example, nitrobenzene was considered to consist of two adsorbing groups, the nitro group and the benzene nucleus. Therefore, equation (16) was written

$$0.68 = (K_{NO_2})(K_{Ph})$$

m-Dinitrobenzene has two nitro groups and one benzene ring, and consequently equation (16) was written

$$2.2 = (K_{NO_2})^2 (K_{Ph})$$

It was assumed here that the different numbers of hydrogen atoms present on the different benzene nuclei would have only a second order-effect on the value of the adsorption affinity of the phenyl group.

A list of the K_{Ph} values calculated in this fashion and the compounds used for their calculation is [illegible] in Table IV. Only those compounds were chosen in which

TABLE IV

CALCULATED PHENYL ADSORPTION AFFINITIES

Compounds	K_{Ph}
Nitrobenzene, *m*-dinitrobenzene	0.21*
Nitrobenzene, *sym*-trinitrobenzene	0.28*
Nitrobenzene, benzaldehyde, *m*-nitrobenzaldehyde	0.23*
Nitrobenzene, *p*-nitrobiphenyl	0.61
m-Dinitrobenzene, *sym*-trinitrobenzene	0.61
Phenol, hydroquinone	0.19*
Phenol, benzaldehyde, *p*-hydroxybenzaldehyde	0.21*
Anisole, *p*-dimethoxybenzene	0.028
Anisole, benzaldehyde, *p*-methoxybenzaldehyde	0.036
Anisole, *p*-methoxybiphenyl	0.98
Anisole, *sym*-trimethoxybenzene	0.07
m-Dimethoxybenzene, *sym*-trimethoxybenzene	0.87

* Chosen as standards for the present work. See text.

the additional geometric and electronic terms were expected to make a minimum contribution to the adsorption affinity of the molecule (*i.e.*, the more strongly adsorbed of a pair of isomers of the compounds in which enhanced resonance effects were least likely were chosen).

The average K_{Ph} value of the standards chosen in Table IV is 0.22. Although it is not entirely clear why some of the other K_{Ph} values should deviate so much, it is possible to attempt some rationalization in terms of geometric factors. However, it must be remembered that a variation even by a factor of seven in K corresponds to a free energy difference of only 1 kcal and consequently it is not surprising that the complexities of the adsorption system result in some variation in the apparent K_{Ph}. Fortunately, the precise value chosen for K_{Ph} has only a minor effect on the results; if the average of all the values in Table IV (0.36) had been chosen instead, it would merely have shifted every individual functional group adsorption affinity by a constant factor and would not have affected the relative values at all. The difference in free energy corresponding to these two K-values, 0.22 and 0.36, is less than 0.3 kcal.

The adsorbing group K-values listed in Table V were calculated with equation (16) from the K_{Ph} value and the K-values for all the monosubstituted benzenoid compounds listed in Table III (except for anisole, the adsorption affinity of which appears to be anomalous). For the determination of the methoxy group value *p*-dimethoxybenzene was chosen. When a di- or polysubstituted compound is used as a standard, an additional geometric factor may enter into the adsorption affinity. Because the internuclear oxygen distance in *p*-dimethoxybenzene is less than the most favorable interadsorbing atom distance of 6.1–6.2 Å (see previous section) the geometric term presumably introduces a small negative error in the $K_{\mathrm{OCH_3}}$ calculation.

TABLE V

GROUP ADSORPTION AFFINITIES AT 24° ± 1°

Adsorbing group	K	σ_K
Acetyl	0.7×10^2	0.1×10^2
Amino (aliphatic)	0.7×10^4	0.2×10^4
Amino (aromatic)	0.8×10^2	0.15×10^2
Carboxyl	3.7×10^2	0.8×10^2
Formyl	23	6
Hydroxyl (aliphatic)	2.6×10^2	0.6×10^2
Hydroxyl (aromatic)	27	5
Methoxyl*	6.9	0.6
Nitro	3.1	0.6
Phenyl	0.22	0.04

* Calculated from *p*-dimethoxybenzene (see text).

The values in Table V express quantitatively the relative order of adsorption affinities of functional groups in the benzene–silicic acid system. The order of affinities is, of course, identical with the qualitative order listed in the previous section. Since

K-values greater than unity mean that the group decreases the free energy of adsorption, all the groups in Table V except phenyl increase the adsorption affinity of a molecule in the benzene–silicic acid system.

The observed K-values for all the compounds listed in Table III which were not used as standards in Tables IV and V are compared in Table VI with the K-values calculated from the data in Table V. The corresponding observed and calculated R°-values are also listed. The agreement between the calculated and observed values is on the whole quite satisfactory considering that a 1000-fold range in K (more than 100-fold in R) is covered. The maximum error in K is a factor of 3, corresponding to about 0.6 kcal in free energy. It is to be noted that the comparison of R-values is a less stringent test.

TABLE VI

CALCULATED AND OBSERVED ADSORPTION AFFINITIES AND CORRESPONDING R°-VALUES*

Compound	K_c	K_o	K_c/K_o	R_c°	R_o°
Anisole	1.5	0.538	2.8	0.40	0.65
m-Dimethoxybenzene	10	9.8	1.0	0.091	0.091
p-Dinitrobenzene	2.1	1.80	1.2	0.32	0.36
m-Hydroxybenzaldehyde	1.4×10^2	93	1.5	0.007	0.011
m-Methoxybenzaldehyde	35	35	1.0	0.028	0.028
p-Methoxybenzaldehyde	35	75	0.5	0.028	0.013
p-Methoxybiphenyl	0.33	0.53	0.6	0.75	0.65
m-Nitroaniline	55	18.0	3.0	0.018	0.053
p-Nitroaniline	55	21.8	2.5	0.018	0.043
p-Nitrobenzaldehyde	16	15.0	1.1	0.059	0.063
p-Nitrobiphenyl	0.15	0.412	0.4	0.87	0.71
Resorcinol	1.6×10^2	164	1.0	0.006	0.006
sym-Trimethoxybenzene	72	33	2.2	0.014	0.029

* The subscripts *c* and *o* refer to calculated and observed values respectively. The system is benzene-heated silicic acid.

In conclusion, the above-described method for analyzing the adsorption affinity of a compound in terms of one parameter for each functional group yields satisfactory approximations that, at least in this system, are on the whole far better than those calculated by means of the empirical multi-parameter LeRosen equation[4]. It is felt that the present approach is sounder and offers promise for investigations of the relationships between the structure of a molecule and its adsorption affinity in other solvent–adsorbent systems as well.

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SUMMARY

The development rates of a variety of monofunctional and polyfunctional aromatic compounds dissolved in benzene have been measured on columns of activated silicic acid–Celite under conditions which at least closely approximate equilibrium. From these rates, extrapolated to the linear region of the isotherm, equilibrium constants for the adsorption process ("adsorption affinities") have been calculated by least squares methods. It is shown that the adsorption affinities of simple polyfunctional compounds can be predicted with reasonable accuracy from individual group adsorption affinities deduced from studies on monofunctional compounds. Quantitative values for the adsorption affinities of some common functional groups in the silicic acid–benzene system are listed.

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A NEW AUTOMATIC RECORDING DENSITOMETER FOR PAPER CHROMATOGRAPHY

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INTRODUCTION

A versatile instrument of high sensitivity for the quantitative estimation of substances on paper chromatograms, which would be capable of dealing quickly with large numbers of such chromatograms and give a permanent record of the results, is highly desirable.

HACKMAN AND LAZARUS[1] have recently discussed the types of densitometers reported in the literature, but none of these seemed to fulfil our requirements.

The advantages of the instrument to be described are that it uses a fluorescent lamp as a light source and it maintains a direct spatial relationship between the chromatogram and the chart record. The fluorescent lamp gives an even illumination and the lack of heat enables the light to be mounted in contact with the paper. The relatively high output of light in the blue region from this type of lamp ensures a good contrast for red spots; lack of blue sensitivity being a disadvantage of incandescent lamps, particularly if used in conjunction with a photocell having an S1 response. The high electrical sensitivity and stability of the instrument enabled us to dispense with the use of dimethyl phthalate for making the paper translucent.

INSTRUMENTATION

The instrument consists of three basic parts (Fig. 1):

1. Light source and photocell assembly;
2. Amplifier and driver unit;
3. Chart recorder.

1. *The light source and phototube unit* (A) consists of a 15 watt 9 inch fluorescent tube, with the phototube (B) arranged parallel to the lamp and just above it. The phototube (type 90 AV with S4 spectral response) is housed inside two concentric brass cylinders each having a 4 mm slit cut longitudinally; the slit being adjustable by rotating the inner cylinder using a reduction drive and a graduated knob (C). The balancing phototube is under the light and is arranged similarly to the top housing.

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* General Motors Holden Fellow.

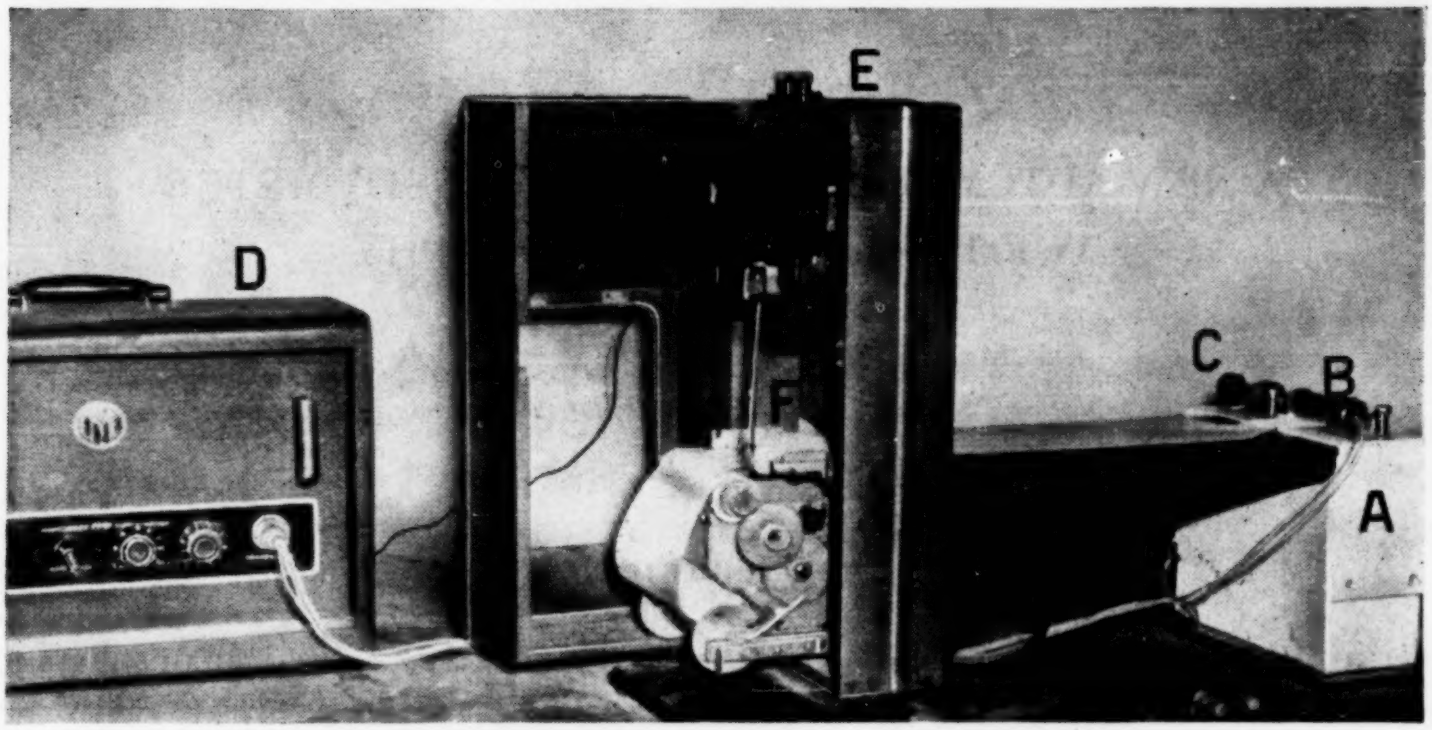
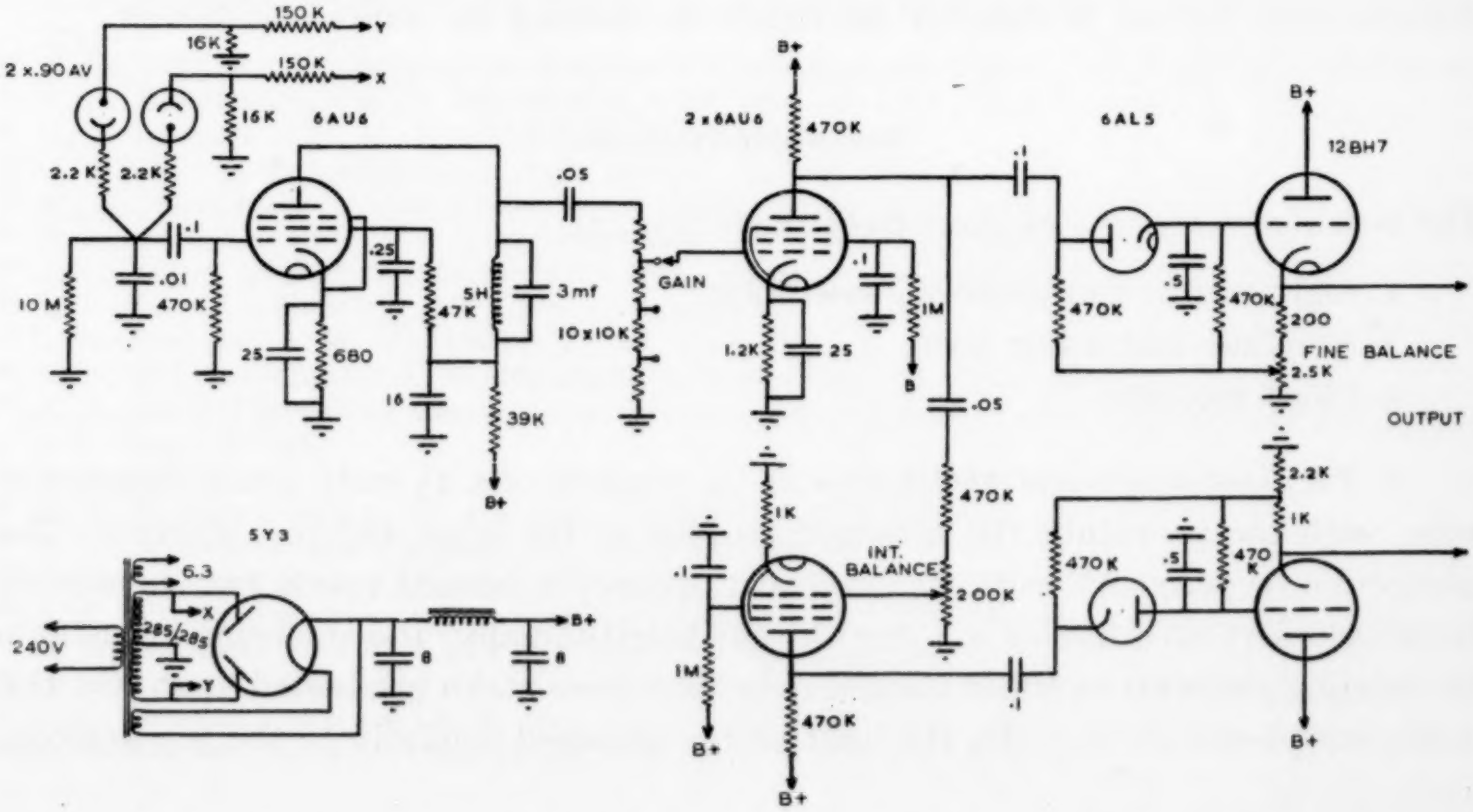


Fig. 1. Automatic recording densitometer.

Coarse balancing is effected by adjusting the slit width. The paper is kept in place by guides 2 inches apart and a roller which prevents buckling. The photocell has a sensitive length of about 2.5 cm; our usual spot width being about 1.5 cm, accurate centralisation of the spot is not necessary.

2. *Amplifier and driver unit* (*D*). This consists basically of a bridge circuit comparing the impedance of the two photocells, a tuned amplifier, detector and driver stage for the chart recorder (Fig. 2).

The rectified 50 c/s signal from the photocells is developed across the 10 megohm resistor. The output at this point is heavily filtered with the 0.01 μF condenser to


150 K
Y
16K
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X
2 x.90 AV
16K
6AU6
2.2 K
2.2K
.1
10 M
.01
470K
.25
25
680
16
47K
5H
3mf
.05
GAIN
10x10K
39K
B+
B+
2x6AU6
470K
.1
1M
B
1.2K
25
.05
470K
INT. BALANCE
200K
1K
.1
1M
B+
470K
B+
6AL5
.1
470K
.5
470K
B+
12BH7
200
FINE BALANCE
2.5K
OUTPUT
2.2K
1K
470K
.5
470 K
.1
B+
5Y3
6.3
X
285/285
Y
240V
B+
8
8

Fig. 2. All capacitances are in microfarads, all resistances are in ohms, kiloohms or megohms.

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reduce interference from the light source which is modulated by about 10% and exhibits a very spiky waveform. The output is fed into an amplifier tuned to slightly below 50 c/s. It is then further amplified in the phase splitter, detected and the rectified signal filtered and passed to the grids of the driver stage. A shift voltage is added at this point to give a fine balance.

This circuit is only one of many possible and is the result of modification of an existing instrument. However, it has proved trouble-free and very stable.

3. *Chart recorder.* A standard dual speed, 5 mA Evershed and Vignoles bench chart recorder (E) was used with a slot (F) cut in its back level with the platen. A shelf was extended out from the back with the light source and phototube assembly mounted at its far end.

A length of chart slightly longer than the chromatograph paper is cut and the chromatogram stapled to one end. The chromatograph paper is fed back between the phototube and light source and the chart fed through the back of the recorder and over the drive wheels.

The recorder pulls the chart under the pen at a speed of 6 inches per minute, at the same time pulling the chromatogram over the light source. This system obviates any slip or mismatch between the chromatogram and the chart record.

The instrument can be adapted for radio chromatograms by replacing the light source and phototube unit with Geiger-Müller tubes and connecting the recorder to the output of a ratemeter. For use in this connection the slow drive on the recorder is used.

APPLICATION

Quantitative estimation of amino acids and related compounds

All standards were chromatographically pure, and reagents (B.D.H.) were of L.R. and A.R. standard. Whatman No. 1 Chromatography Paper was used for most determinations, though other grades of paper could also be used. This was ruled with a pencil so that the starting line was 1 ½ inches from the bottom of the paper and the width was divided into 2 inch wide "strips". These were separated after application of the detecting reagent. At least three such strips were run simultaneously in the one determination. They were spotted either with three standards at different concentrations or with one standard, a control and a test solution.

The spot was applied with a "Shandon" pumpette fitted with a suitable "Shandon" micropipette. Various solvent systems have been used. However, it was found that for the estimation of amino acids in toad sartorii, *n*-butanol–*n*-propanol–0.05 *M* HCl (1:2:1) gave a clear separation by the ascending technique in 16–20 h. The chromatograms were dried in a draught oven at 50° for 30 min. The paper was then dipped in a solution of ninhydrin in acetone, to which pyridine had been added[2]. It was found that this method was necessary to produce a constant colour especially in the case of the acid solvents. KAY *et al.*[3] have discussed the "quantification" of the ninhydrin reaction and have used a caustic spraying mixture to give the alkaline reaction. We have found that under these conditions the spray damages the paper rendering subsequent techniques difficult and unreliable. The paper was then heated again at 50° for 30 min. This produces even and reproducible colours. The paper was cut in two inch strips and scanned as described with the densitometer. Fig. 3 shows a typical tracing obtained during an investigation into the effects of abnormal constituents of Ringer on the excised muscle of the toad, *Bufo marinus*. Here we see that addition of large amounts of caesium has caused a breakdown in the carnosine and glutamine with a consequent increase in histidine, β-alanine and glutamic acid.

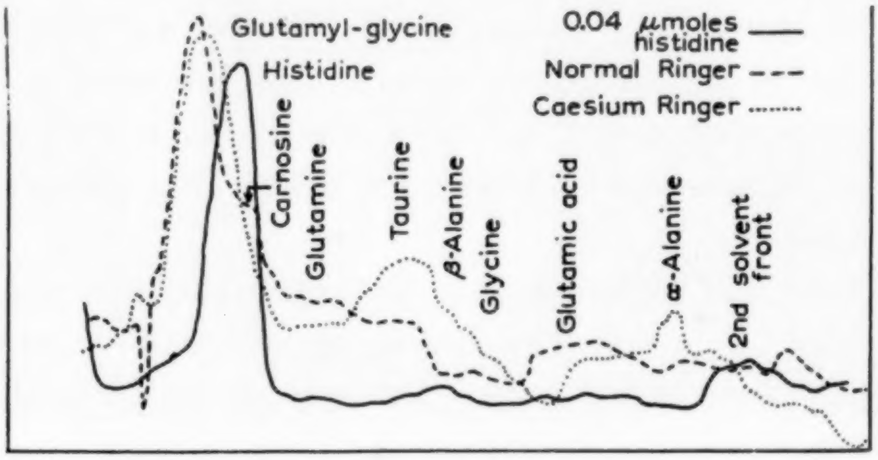

Fig. 3. Typical recording.

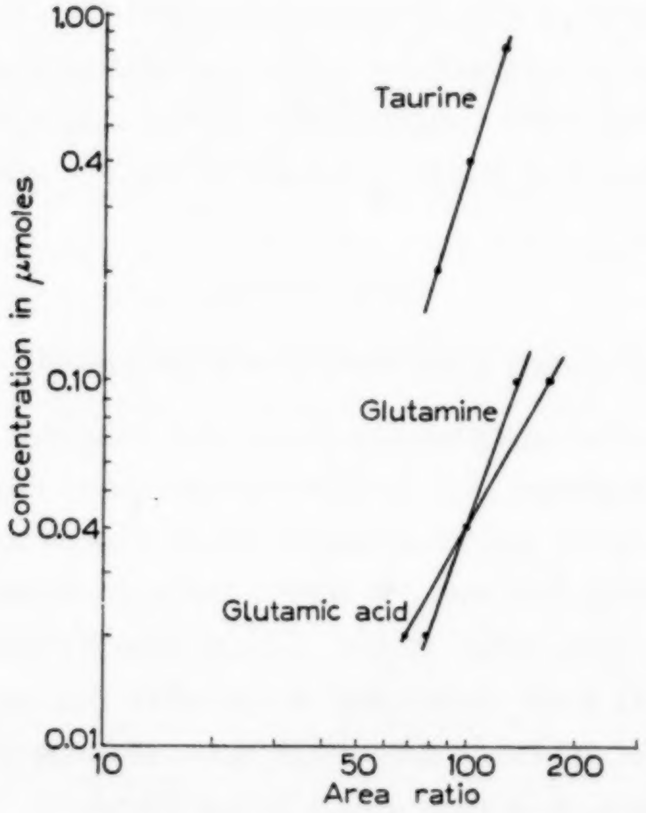

Fig. 4.

For well defined peaks such as glutamyl-glycine and histidine in Fig. 3, measurement of areas from the pen recording gave a quantitative estimate of material on the paper scanned. The standard, control and test were recorded on the same chart in different colours. For smaller quantities it was not always possible to investigate new solvents to give a sharper separation; therefore, providing a reasonable separation was obtained, a "carrier technique" was used. This consisted of adding the same quantity of standard to both the control and test samples; and allowing for that standard in reading the final result. By this method, between 0.005 and 0.040 μmoles of most amino acids could be estimated. When no clear separation occurred, the balance of the curve was drawn by hand[4].

The area under the curve was estimated with an "Allbrite" planimeter, which gave the area in arbitrary units. It was found that variations in the colour produced through deviations from the detection procedure could be compensated by running a standard, whose area was designated as 100, with each paper.

Typical calibration curves are shown in Fig. 4. It will be seen that a plot of the area against the concentration on log–log paper produces a linear calibration. The accuracy of the method is ± 3%.

Other uses

The densitometer has also been used for quantitative estimation of the red colour produced by the reaction of carnosine, histamine and histidine with diazotized sulphanilic acid reagent.

The method of Ames and Mitchell[5] was modified to eliminate the sodium carbonate spray, by introducing liquid ammonia into the draught chamber and allowing for full colour development. Purines also gave linear calibrations when the photograph obtained with ultraviolet photography was submitted to the scanning technique. Full details of this method are described by us in another paper[6].

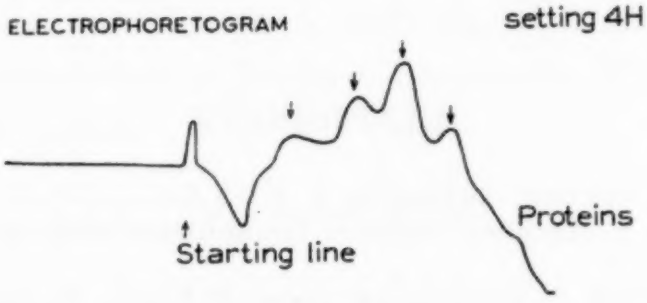

Fig. 5.

The scanning of photographs of starch electrophoretograms has been undertaken by Miss Helen Silberman of the Biochemistry Department. Clear records have thus become available for the estimation of proteins by this technique.

Semi-quantitative results were also obtained for molybdate blue in phosphate estimations and for proteins separated by electrophoresis and detected with bromphenol blue in saturated mercuric chloride solution[7]. A typical record is shown in Fig. 5.

CONCLUSION

The instrument has the following advantages over other models; it has a high sensitivity, a positive one-for-one spatial correspondence between chromatogram and chart, and does not heat the paper.

The densitometer described has proved highly satisfactory in use over the past

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year in the evaluation of a great variety of substances. It has proved useful qualitatively in precise determinations of the R_F values of amino acids, phosphates, purines, nucleotides, proteins and alkaloids and has been used in estimation of amino acids.

The instrument has a wide range of possible applications which covers colorimetric, photographic and radiometric techniques, and should find extensive use in industry and research.

ACKNOWLEDGEMENT

The authors wish to acknowledge the help and encouragement of Professor F. H. SHAW and financial assistance from the National Health and Medical Research Council of Australia and General Motors Holden.

SUMMARY

An automatic recording densitometer is described which has high sensitivity and eliminates the need for making the paper translucent. It can be adapted to scan chromatograms using colorimetric, photographic or radiometric techniques. It provides a positive 1:1 correspondence between record and paper and has an overall accuracy of better than ± 3 %.

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INFLUENCE OF THE SUPPORTING MEDIUM ON THE FRACTIONATION OF PROTEINS BY ZONE ELECTROPHORESIS*

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Since various supporting media used for zone electrophoresis each offer certain advantages and limitations[1-3], an attempt was made to combine two different media[4]. During the course of this work, it became apparent that the results of the fractionation of mouse plasma were significantly different when protein was allowed to migrate in a medium of potato starch granules or in one of cornstarch gel. Therefore, the two-dimensional technique of electrophoresis[5-7] was used to obtain increased insight into the mechanism of the separation. The following supporting media were studied: (a) a slab of potato starch granules, (b) a cornstarch gel stiffened by the addition of amylose, and (c) the combination of the two. The results of these experiments will be described in the present paper.

EXPERIMENTAL

Starch electrophoresis

Experiments were carried out in a 30 × 5 × 1.3 cm plastic tray. Strips of Whatman No. 3 filter paper were used to connect the two ends of the tray to buffer vessels, which in turn were connected by paper strips to vessels containing silver–silver chloride electrodes. All experiments were carried out at an ionic strength of 0.05 in veronal citrate buffer of pH 8.6 or 7.4 or phosphate buffer of pH 7.0.

The technique of using potato starch granules was based upon the method described by KUNKEL AND SLATER[8]. For most experiments, the potato starch was extensively washed by first allowing the granules to settle from a suspension with two volumes of buffer, after which the wet granules were further washed on a funnel by dripping through them five volumes of buffer over a period of several hours. Some samples of potato starch were washed with dilute ammonium or sodium hydroxide, then with water and were finally dried in a desiccator**.

Zone electrophoresis on a supporting medium of conrstarch gel was performed according to BERNFELD AND NISSELBAUM[9]. A combination of the two media was achieved by fitting a cardboard or wooden form covered with aluminum foil in the center of the tray leaving 3.5 cm wide sections at each end. After the starch paste was poured

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[**] Starch washed with alkali obtained through courtesy of Dr. GAIL L. MILLER.

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into the ends and allowed to gel overnight in the cold, the form was removed, and the center section was filled with potato starch granules.

The progress of the electrophoretic separation was observed under ultraviolet light, and the electric field was maintained until the albumin fraction had moved 9–10 cm away from the γ-globulin fraction.

A 3 mm × 3 mm longitudinal segment was then removed from the center of the block and applied to a sheet of Whatman No. 1 filter paper. A filter paper electrophoresis at pH 8.6 was then carried out in an electric field perpendicular to the one in the starch medium.

Filter paper electrophoresis

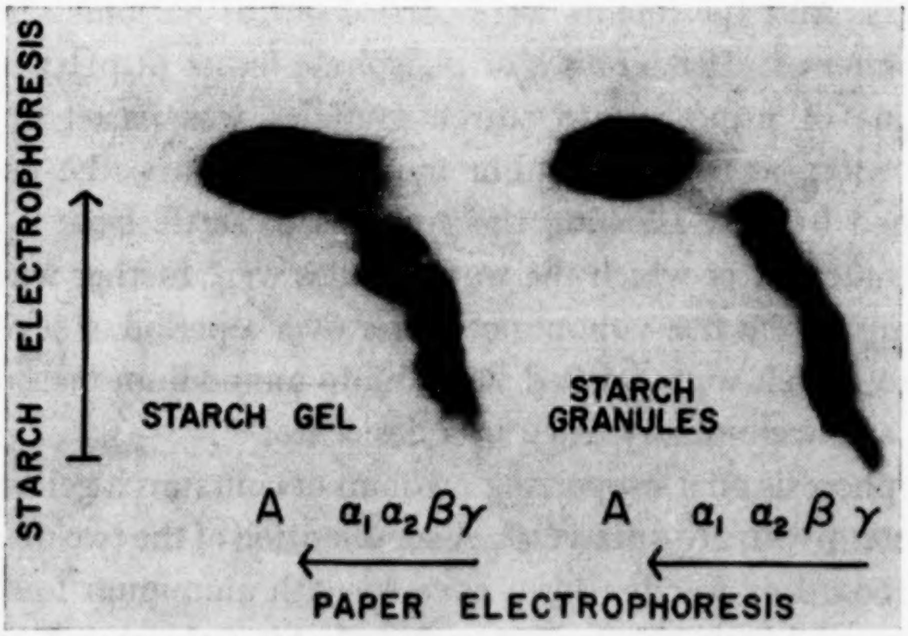
Experiments were carried out in a horizontal strip apparatus* with 0.1 ionic pH 8.6 veronal citrate buffer. After applying a current of 16–18 mA for 1–1½ to 2 h, the starch segment was removed from the paper. Electrophoresis on paper was then continued for 3 to 4 h at 16–18 mA, or overnight at correspondingly lower field strength. Bromphenol blue was used for staining the protein.

Plasma from normal or from tumor (Sarcoma I)-bearing mice of the A/Jax inbred strain was used. Tumor-bearing animals were bled 10 to 12 days after the implantation.

RESULTS

Influence of the medium

When the mouse plasma was separated by electrophoresis on filter paper in pH 8.6 veronal citrate buffer, the five common components were resolved; and the pattern was similar to that of a pool of human plasma. Since the mouse plasma was from an inbred strain of animals, the pattern was quite reproducible, and samples from different


STARCH ELECTROPHORESIS
STARCH GEL
STARCH GRANULES
A $\alpha_1 \alpha_2 \beta \gamma$
A α_1 α_2 β γ
PAPER ELECTROPHORESIS

Figs. 1a and b. Two-dimensional electrophoresis of mouse plasma in a starch medium (vertical direction) and on paper (horizontal direction) both in veronal citrate buffer at pH 8.6; (a) in cornstarch gel, (b) in potato starch granules.

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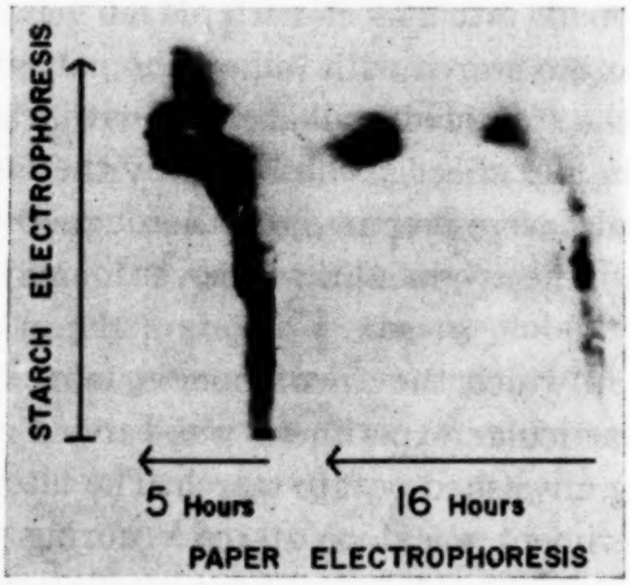
* Purchased from Laboratory Glass Instrument Corp., New York.

animals were not subject to the variations found with human plasma from different individuals.

The results obtained by two-dimensional electrophoresis in veronal citrate buffer pH 8.6 with plasma from tumor-bearing animals are shown in Figs. 1a and 1b. It is evident that the mobility of the α_2-globulins is significantly different in a cornstarch gel (Fig. 1a), from that in a medium of potato starch granules (Fig. 1b). In the former medium the α_2-globulins have a mobility closer to that of the β-globulin, while in potato starch granules, this protein fraction is found nearer to albumin. The mobilities of all the other protein components appeared to be identical in the two starch media. In neither of the starch media at pH 8.6 did the α_1-component separate from albumin, while these two components can easily be separated at this pH on paper electrophoresis.



Figs. 2a and b. Two-dimensional electrophoresis of mouse plasma on a medium of unwashed potato starch granules with sections of cornstarch gel at both ends in veronal citrate buffer; the pH of the buffer (8.6) was uncontrolled because of the lack of washing the medium (see text). Similar patterns are obtained on the same medium equilibrated with buffer of pH 7.0.

The medium consisting of a combination of starch gel and starch granules gave a pattern identical with that of potato starch granules alone. However, one striking difference was noted; that is, the absence of electro-osmotic flow. In this respect, the fractionation on the combined media resembled that obtained on the cornstarch gel.

The effect of the supporting medium on pH

In both starch media at pH 7.0 in phosphate buffer a component was found to move ahead of albumin, while no such fast protein fraction was found in these media at pH 8.6 in veronal citrate buffer as was shown in Fig. 1. From the filter paper pattern of the two-dimensional experiment (like that shown in Fig. 2), this component is clearly identified as an α_1-globulin, since it appears in its normal position between albumin and the α_2-globulins on paper at pH 8.6. A similar pattern was found in the starch media in veronal citrate buffer of pH 7.4, which shows that the phenomenon was not due to the phosphate ions of the buffer.

The reversal of the mobilities of albumin and α_1-globulin on the starch media upon lowering the pH from 8.6 to 7.0 emphazises the prime importance of the pH of the starch media. This is particularly relevant in the case of a medium consisting of potato starch granules, since this material frequently retains a considerable amount of acidity which can only be removed by repeated washings. One lot of potato starch, for instance, was found to lower the pH of distilled water to 4.39. Thus, when certain lots of potato starch were made into a block with pH 8.6 veronal citrate buffer, without previously washing the potato starch with buffer, the pattern was identical with that found at pH 7. Washing the starch with alkali, then with water and drying it was not effective in eliminating the pH effect, as observed by the fast moving α_1-component. When potato starch granules were prepared, by thoroughly washing and equilibrating them with buffer of pH 8.6, the α_1-globulin was never found to move ahead of albumin at this pH.

The type of pattern in which the α_1-component moved ahead of the albumin is shown in Fig. 2a. This particular experiment was carried out on a combination gel potato starch media using unwashed potato starch. The filter paper electrophoresis of the two-dimensional experiment was done at 400 V during 5 h. Fig. 2b was obtained from a segment from the same starch experiment after electrophoresis on filter paper for 16 h at 180 V. The latter shows more clearly than Fig. 2a that the component having the same mobility as albumin on potato starch granules appears as an α_2-globulin on filter paper. The component which moved ahead of the albumin spread on prolonged paper electrophoresis so that it does no longer show in Fig. 2b, although it was faintly visible on the original filter paper.

DISCUSSION

The results have shown a significant difference between the mobilities of the α-globulins on potato starch granules and those on a cornstarch gel. Differences between the fractionation of the hemoglobin-binding globulins on potato starch granules and a potato starch gel have also been found by BEARN AND FRANKLIN[10]. While it thus appears established that plasma fractionation is influenced by the nature of the supporting medium, our results have further shown that a combination of the two starch media retains some of the characteristics of each. The combined medium has all the advantages of starch granules with respect to easy elution without having the disadvantage of the electro-osmotic flow. The absence of electro-osmotic flow in stiff starch gels has been previously demonstrated[9], and the mechanism involved here is probably mechanical hindrance of flow. The prevention of electro-osmotic flow facilitates location of proteins according to electrophoretic mobility, and a combination of gel with other media may be quite useful, for example in the fractionation of carbohydrate on polyvinyl chloride[11] which has been observed to have an excessively high electro-osmotic flow rate[12].

In addition, our results have demonstrated that the medium itself may, in the case of potato starch granules, affect the fractionation by altering the pH of the buffer solution, obviously due to the presence of acidic impurities. This effect could be eliminated by thoroughly washing and equilibrating the starch granules with the buffer solution, but not by washing with dilute alkali and H_2O. This emphazises the necessity of proper preparation of the potato starch granules. KUNKEL[1] has recommended the use of warm buffer to wash potato starch.

In 1942 SEIBERT and coworkers[13, 14] using the moving boundary technique, first described a component moving faster than albumin in pH 7.7 phosphate buffer and suggested that this might be the α-component which LONGSWORTH had found to move more slowly than albumin in veronal buffer at pH 8.6[15]. Since then many workers have described "prealbumins" in various types of electrophoretic separations[5, 7, 16–22] and some have suggested their association with plasma mucoproteins. Our results have shown quite clearly that the component moving ahead of albumin on both cornstarch gel and potato starch granules is the α_1-globulin. This behavior of α_1-globulin is no doubt a different phenomenon from some of the observations of "prealbumin" components which have been found to occur in small amounts in human serum at pH 8.6.

SUMMARY

1. The fractionation of mouse plasma by zone electrophoresis on (a) potato starch granules, (b) cornstarch gel and (c) a combination of the two media has been compared by two-dimensional electrophoresis with filter paper electrophoresis as the second stage.

2. A significant difference between the two starch media was observed in that α_2-globulins migrate in a cornstarch gel more closely to the β-globulin than they do in potato starch granules. The component which appears as α_1-globulin on filter paper electrophoresis does not separate from the albumin on either starch medium at pH 8.6 and migrates ahead of the albumin at pH 7.0.

3. A combination of potato starch granules with sections of gel at the electrode ends yields the type of fractionation identical with that of potato starch granules alone, but prevents electro-osmotic flow, thus combining the advantages of both media.

4. Potato starch granules may alter the pH in the supporting medium, and, hence, markedly influence the type of fractionation. Only thorough washing and equilibrating the potato starch with buffer resulted in reproducible results with different lots of potato starch and yielded types of fractionation compatible with the respective pH of the buffer.

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BEITRÄGE ZUR CHEMIE UND PHYSIOLOGIE EINIGER STOFFWECHSELCHEMISCH WICHTIGER SÄUREN

3. MITTEILUNG.

EIN EINFACHER PAPIERCHROMATOGRAPHISCHER NACHWEIS FÜR ISOCITRONENSÄURE IN PFLANZENMATERIAL*

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Unsere lückenhaften Kenntnisse über die Verbreitung der Isocitronensäure in pflanzlichem Material sind im wesentlichen in dem Mangel an einfachen und sicheren Methoden zum Nachweis und zur Bestimmung dieser stoffwechselchemisch wichtigen Säure begründet.

Nachdem es bislang nicht gelungen war, Isocitronensäure von Citronensäure als freie Säure papierchromatographisch zu trennen, standen neben der Isolierung der Säure[2,3] lediglich sehr zeitraubende Verteilungsverfahren an Silicagel-Säulen[4–7], das enzymatische Verfahren von KREBS UND EGGLESTON[8] in der Modifikation von HARGREAVES, ABRAHAMS UND VICKERY[9] sowie eine von RANSON[10] beschriebene indirekte papierchromatographische Methode zur Verfügung. Letztere beruht darauf, die Isocitronensäure durch trockenes Erhitzen im Vakuum in Isocitronensäurelakton überzuführen und mit konzentrierter Ammoniakflüssigkeit als "Isocitronensäureamid" zu lösen. Diese Verbindung zeigt in den meisten Verteilungsmitteln weit höhere R_F-Werte als Citronen- und Isocitronensäure. Da der Nachweis in Säuregemischen durch andere Komponenten gestört werden kann, schneidet man aus einem ersten direkt gewonnenen Chromatogramm Isocitronen- und Citronensäure als gemeinsamen Fleck aus, führt die Umwandlung der Isocitronensäure in das "Amid" mit dem Eluat des Chromatogrammfleckes durch und chromatographiert erneut. Diese Arbeitsweise ist ebenso kompliziert.

Wir gingen bei unseren Versuchen zur direkten papierchromatographischen Trennung von Isocitronen- und Citronensäure als freie Säuren von der Beobachtung aus, dass die beiden Säuren in einigen Verteilungsmitteln kleine R_F-Wert-Unterschiede aufweisen (Tabelle I).

Weitere in dieser Richtung durchgeführte Untersuchungen ergaben, dass die geringe R_F-Wert-Differenz (Diff. $R_F \times 100 = 2$) bei gleichzeitig stark herabgesetzten R_F-Werten, vor allem mit dem von uns schon früher zur quantitativen Abtrennung der Äpfel- von der Citronensäure benutzten Verteilungsmittel Isoamylalkohol–Chloroform–Ameisensäure 85% (4:1:1 v/v/v), wassergesättigt, unter geeigneter papierchromatographischer Technik zur reproduzierbaren Trennung von Isocitronen- und Citronensäure in Modellgemischen und in Pflanzenextrakten ausreicht (Tabelle II).

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* 2. Mitteilung, siehe Ref.[1].

Literatur S. 529/530.

TABELLE I

R_F-WERTE VON ISOCITRONENSÄURE UND CITRONENSÄURE IN EINIGEN VERTEILUNGSMITTELN

Verteilungsmittel	*Methode*	$R_F \times 100$	
		Isocitronensäure	*Citronensäure*
tert.-Amylalkohol–Chloroform–Ameisensäure 90%–Wasser (80:80:30:80 v/v/v/v[11])	Absteigend	24	26
tert.-Amylalkohol–Chloroform–Ameisensäure 90%–Wasser (24:136:30:80 v/v/v/v[11])	Absteigend	10	11
n-Butanol–Chloroform–Ameisensäure 10% (75:25:10 v/v/v[12])	Rundfilter	27	26
Isoamylalkohol–Chloroform–Ameisensäure 85% (4:1:1 v/v/v) wassergesättigt[13])	Absteigend	12	14

TABELLE II

PAPIERCHROMATOGRAPHISCHE TRENNUNG VON ISOCITRONENSÄURE UND CITRONENSÄURE IM VERTEILUNGSMITTEL ISOAMYLALKOHOL–CHLOROFORM–AMEISENSÄURE 85% (4:1:1 v/v/v), WASSERGESÄTTIGT

(Mittelwerte aus 20 Versuchen)

Verfahren	*Absteigend mit Front ca. 40 cm*	*Absteigend ohne Front*	*Durchlaufend, Laufzeit: 6 Tage*	
Ermittelte Grössen	$R_F \times 100$	$R_{Weinsäure} \times 100$	"R_F" × 100 *berechnet*	*Wanderungsstrecke, Weinsäure = 15 cm*
Spalte	*1*	*2*	*3*	*4*
Weinsäure	8	100	8.0	15 cm
Phosphorsäure	8	105	8.4	15.8 cm
Isocitronensäure	12	163	13.0	24.5 cm
Citronensäure	14	183	14.6	27.5 cm
Äpfelsäure	23	D	D	D

"R_F" = Aus Spalte 2 berechnete theoretische Wanderungsweite der Lösungsmittelfront (Durchschnitt 202 cm).

D = Die Säure wandert meist in den Durchlauf des Chromatogramms.

Nach unseren Versuchsbedingungen wandert die Weinsäure als Bezugssubstanz während 6 Tagen zwischen 15 und 20 cm, was einem Abstand der Mittelpunkte des Isocitronensäure- und des Citronensäureflecks von 3 bis 4 cm entspricht. Damit ist eine einwandfreie Trennung im Bereich der papierchromatographischen Möglichkeiten (etwa 40–400 μg) gegeben. Eine Verwechslung mit anderen nichtflüchtigen Säuren kommt bei unserem Verfahren kaum in Betracht, da die in ihrer Wanderungsweite am nächsten liegenden Säuren von allgemeiner Verbreitung (Phosphor- und

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Äpfelsäure) sehr weit abgetrennt werden (Tabelle II). Zur Erhärtung der Ergebnisse kann jedes Chromatogramm mehrfach wiederholt werden, wobei man die Doppel zur Ausführung von Farbreaktionen, die von Isocitronen- und Citronensäure in gleicher Weise gegeben werden, benutzt.

Dazu empfehlen sich Farbreaktionen, z.B. nach BUCH, MONTGOMERY UND PORTER[14]. Man besprüht mit einer frisch bereiteten Mischung von Essigsäureanhydrid und wasserfreiem Pyridin (1:9 v/v) und stellt nach 3 Minuten im Tageslicht orangefarbene Flecke fest, oder besprüht auf einem anderen Chromatogramm nach SCHREIER UND HACK[15] mit einer 4%igen Lösung von *p*-Dimethylaminobenzaldehyd in Essigsäureanhydrid mit Zusatz einer kleinen Menge Natriumacetat, erhitzt das Chromatogramm 2 Minuten bei 140° und erhält im Tageslicht weinrote Flecke.

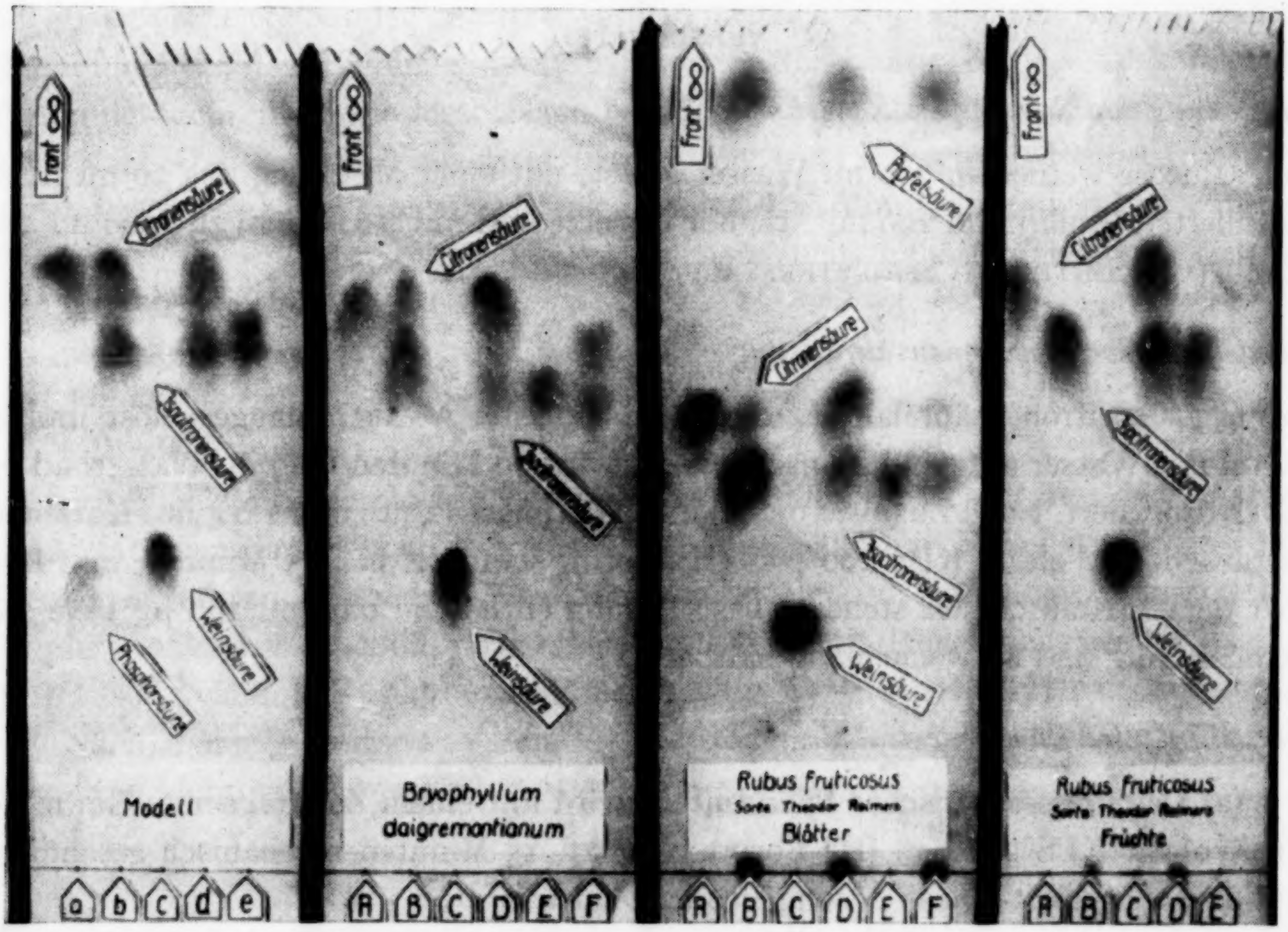
Front
Citronensäure
Isocitronensäure
Weinsäure
Phosphorsäure
Modell
a
b
c
d
e
Front
Citronensäure
Isocitronensäure
Weinsäure
Bryophyllum daigremontianum
A
B
C
D
E
F
Front
Äpfelsäure
Citronensäure
Isocitronensäure
Weinsäure
Rubus fruticosus
Sorte Theodor Reimers
Blätter
A
B
C
D
E
F
Front
Citronensäure
Isocitronensäure
Weinsäure
Rubus fruticosus
Sorte Theodor Reimers
Früchte
A
B
C
D
E

Fig. 1. Papierchromatographischer Nachweis von Isocitronensäure. a = Phosphorsäure + Citronensäure; b = Isocitronensäure + Citronensäure; c = Weinsäure; d = Isocitronensäure + Citronensäure; e = Isocitronensäure; A = Citronensäure; B = Pflanzenextrakt + Isocitronensäure; C = Weinsäure; D = Pflanzenextrakt + Citronensäure; E = Isocitronensäure; F = Pflanzenextrakt.

Die beschriebene papierchromatographische Methode wurde von uns zum Nachweis von Isocitronensäure in verschiedenen Pflanzenextrakten angewandt. Einige Originalchromatogramme sind in der Fig. 1 wiedergegeben. Danach kommt in den Blättern von *Bryophyllum daigremontianum* Berger sowohl Isocitronen- als auch Citronensäure vor. Erstere wurde in dieser Pflanze von PUCHER UND VICKERY[16] nachgewiesen. Grosse Mengen Isocitronensäure, aber keine Citronensäure in der gleichen Grössenordnung, fanden wir in den Früchten von *Rubus fruticosus* L.

(Sorte Theodor Reimers). Aus Brombeeren wurde die Isocitronensäure bereits von NELSON[2] isoliert. Im Gegensatz zu früheren Angaben[8], nach denen in den Blättern von Pflanzen der Gattung *Rubus* keine bemerkenswerten Mengen an Isocitronensäure vorkommen sollen, konnten wir in den Blättern der Brombeer-Gartensorte "Theodor Reimers" mehr Isocitronen- als Citronensäure finden. In den Blättern einiger Wildarten der Gattung *Rubus* war Isocitronensäure allerdings papierchromatographisch nicht nachweisbar.

Über quantitative Untersuchungen an den Blättern solcher *Rubus*-Wildarten, die Isocitronensäure in erheblichen Mengen führen, wird zu gegebener Zeit noch zu berichten sein.

VERSUCHSTEIL

Herstellung von Schweppe's Reagens[17]

20 g Glucose werden in 200 ml Wasser gelöst, mit einer Mischung aus 20 ml frisch destilliertem Anilin und 200 ml Äthanol versetzt und mit *n*-Butanol zu 1000 ml aufgefüllt (vgl. SMITH UND SPRIESTERSBACH[18]).

Herstellung der Isocitronensäurelösung

50 mg *dl*-Isocitronensäurelakton werden in 1.5 ml 1 *N* Natronlauge gelöst und zu 5.0 ml mit Wasser aufgefüllt. Anschliessend wird 60 Minuten lang im Wasserbad bei 40° hydrolysiert. Nach Zugabe von 0.5 ml 1 *N* Ameisensäure und 1.0 g des Kationenaustauschers Wofatit KPS 200 p.a. (H^+-Form) schüttelt man 5 Minuten und lässt über dem Austauschharz stehen. Diese Lösung enthält in 0.01 ml 100 μg freie Isocitronensäure und ist wochenlang haltbar.

Herstellung der Pflanzenextrakte

(a) Der Presssaft frischer Pflanzenteile wird mit einem Zehntel seines Gewichtes mit Wofatit KPS 200 p.a. (H^+-Form) versetzt, 15 Minuten mechanisch geschüttelt und abzentrifugiert. Diese Lösung ist nicht haltbar.

(b) Einige Gramm frisches Pflanzenmaterial werden im Mörser mit 0.5 ml 4 *N* Schwefelsäure zerkleinert und mit etwa 10 g wasserfreiem Natriumsulfat zur Trockne verrieben und im Soxhlet-Apparat 2 bis 3 Stunden mit Aceton extrahiert (bis der Ablauf farblos ist). Das auf 10 ml vorsichtig eingeengte Extrakt ist monatelang haltbar. Wegen der grösseren Verdünnung muss unter Umständen streifenförmig aufgetragen werden.

Papierchromatographische Technik

Auf Filtrierbogen Schleicher & Schüll 2043b mgl (ungewaschen) 18 × 58 cm (Faserrichtung parallel zur Schmalseite) werden auf einer Startlinie, 12 cm von der Schmalseite entfernt, Lösungen von Citronen- und Isocitronensäure als Testsubstanz, von Weinsäure als Bezugssubstanz und das zu untersuchende Pflanzenextrakt (ev. mit wechselweisem Zusatz von Reinsubstanz) mehrfach aufgetragen. Die gegenüberliegende Schmalseite wird mit 1 cm tiefen sägeförmigen Einschnitten versehen. Man hängt

Literatur S. 529/530.

die Bogen zur Sättigung mit dem einphasigen Verteilungsmittel in eine Kammer für absteigende Chromatographie. Auf dem Boden der Kammer befinden sich drei Glasschalen mit je 10 ml des Flüssigkeitsgemisches Isoamylalkohol–Chloroform–Ameisensäure 85% (4:1:1 v/v/v), wassergesättigt, das 12 Stunden vor der Verwendung frisch herzustellen ist. Die Wände der Kammer sind zur besseren Sättigung mit drei mit dem Lösungsmittelgemisch getränkten Filtrierpapierstreifen von 3 cm Breite senkrecht ausgekleidet; sie ragen an ihrem unteren Ende in die Bodenschalen hinein. Sättigungszeit mindestens 16 Stunden. Danach wird das gleiche Verteilungsmittel in die Rinnen gefüllt und 6 Tage absteigend unter Abtropfen des Verteilungsmittels vom gezähnten unteren Rand des Papierbogens chromatographiert. Die Kammern können bis zu 3 × hintereinander benutzt werden. Danach empfiehlt sich eine gründliche Reinigung, da der zunehmende Gehalt der Atmosphäre an Isoamylformiat die Trennung nachteilig beeinflusst. Nach beendeter Chromatographie werden die Bogen über Nacht bei Zimmertemperatur getrocknet, mit Schweppe's-Reagens besprüht und sofort bei 125° im Trockenschrank etwa 5 bis 10 Minuten (je nach Kapazität des Trockenschrankes) erhitzt. Die Säuren erscheinen als nahezu unbegrenzt haltbare braune Flecke auf weissem Grund.

ZUSAMMENFASSUNG

Es wird über ein papierchromatographisches Verfahren berichtet, das den Nachweis der Isocitronensäure neben Citronensäure als freie Säuren in Modellgemischen und in Pflanzenextrakten gestattet. Die Trennung erfolgt im Durchlaufchromatogramm (Papier: Schleicher & Schüll 2043b mgl, 18 × 58 cm) mit dem einphasigen Verteilungsmittel Isoamylalkohol–Chloroform–Ameisensäure 85% (4:1:1 v/v/v) wassergesättigt.

Nach dieser Arbeitsweise konnte Isocitronensäure in verschiedenen Pflanzenextrakten identifiziert werden.

SUMMARY

A paper-chromatographic method is described for the detection of isocitric acid in the presence of citric acid, both as free acids, in synthetic mixtures and plant extracts. The separation is carried out by the descending technique, allowing the solvent to flow off the paper. Schleicher & Schüll paper 2043b mgl 18 × 58 cm, and the monophasic solvent isoamyl alcohol–chloroform–85% formic acid (4:1:1 v/v/v), saturated with water, were used.

With this method, isocitric acid could be identified in various plant extracts.

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PAPER ELECTROPHORESIS OF NICOTINIC ACID DERIVATIVES

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Studies on the metabolism of nicotinic acid and nicotinamide have revealed that the deamidation of nicotinamide constitutes an important aspect of the vitamin metabolism in several organisms and indeed appears to be the major metabolic pathway in the mould *Neurospora crassa*[1] and the insect, *Corcyra cephalonica* St.[2]. The tissues of the pigeon and the chick have also been found to have particularly strong nicotinamide deamidase activity[3]. These earlier studies have involved a preliminary qualitative detection of deamidase activity through the application of the paper chromatographic technique and the subsequent quantitative determination of enzyme activity by a microbiological assay procedure using the organism, *Leuconostoc mesenteroides* 9135[4].

In the course of studies on the properties of the nicotinamide deamidase system in *Neurospora*, it was observed that the enzyme activity was rather unstable[1]. In view of this fact and also owing to the non-availability of a quick and efficient method for the identification of enzyme activity associated with any particular fraction, a purification of the *Neurospora* enzyme could not be effected; the enzyme system in *Corcyra* larval tissue has been found to be equally unstable. Since even the paper chromatographic technique is comparatively slow, the possibility of applying the technique of paper strip electrophoresis to the separation of nicotinic acid and nicotinamide and some of their derivatives was investigated. The results show that the electrophoretic method lends itself for purposes of quick and highly efficient separation of these compounds. The experimental details involved are given below.

EXPERIMENTAL MATERIALS AND METHODS

The paper strip electrophoresis unit marketed by Arthur H. Thomas and Co., Philadelphia (U.S.A.) was used in these investigations. Whatman No. 1 filter paper strips (30 × 4 cm) were first soaked in the appropriate buffer and then dried by pressing between filter paper sheets. The dried strips were supported on the rectangular wooden frame, taking care to avoid sagging, and allowed to equilibrate with the electrolyte for 10 minutes at a potential difference of 280 V.

Aqueous solutions of authentic samples of nicotinic acid, nicotinamide, nicotinuric acid, N′-methylnicotinamide, diphosphopyridine nucleotide (DNP) and triphosphopyridine nucleotide (TPN) were prepared to give a concentration of about 100 µg/ml in each case. 10–20 µl portions of these solutions were applied individually to the strips in the form of a band across the breadth of the strip halfway between the electrodes. A solution of a mixture of all these compounds was also similarly applied. The electrophoresis was carried out at room temperature by the application of a potential difference of 280 V for 1 to 2 hours, after which the ends of the strips were gently pressed with a filter paper pad to remove excess buffer. The filter paper strips were then dried by keeping at 100° for 5 to 10 minutes and examined after suitable treatment for the location of bands of the various compounds. Nicotinic acid, nicotinamide and nicotinuric acid were detected by first exposing the strips to cyanogen bromide vapours for 15 minutes and subsequently spraying them with a 0.25% solution of benzidine in ethanol. A second exposure to cyanogen bromide vapours after the spraying resulted in the formation of a dark red band corresponding to the position of nicotinic acid, a brown red band in the case of nicotinamide and a purple red band in the case of nicotinuric acid. N′-methylnicotinamide DPN, and TPN were located as blue-fluorescing bands in ultraviolet light after exposing the electrophoretogram to vapours of a mixture of ethyl methyl ketone and ammonia.

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The following buffer systems were tested for the effective separation of nicotinic acid and its derivatives: *M*/20 veronal pH 8.6, *M*/20 tris(hydroxymethyl)aminomethane (Tris) pH 9.0, *M*/30 phosphate pH 7.0, *M*/30 phosphate pH 10.2, *M*/30 borate pH 8.9 and *M*/10 acetate pH 4.8.

RESULTS AND DISCUSSION

The pattern of separation of nicotinic acid, nicotinamide, nicotinuric acid, N′-methylnicotinamide, DPN and TPN is presented in Table I as the distance travelled by each compound in 1 hour from the point of application. In all the buffers, under the conditions described, nicotinic acid and nicotinuric acid moved towards the anode, the former being the faster-moving, while N′-methylnicotinamide and nicotinamide moved towards the cathode, nicotinamide having the lesser mobility. DPN and TPN

TABLE I

ELECTROPHORETIC MOVEMENT OF NICOTINIC ACID DERIVATIVES IN DIFFERENT BUFFERS

Expressed in terms of distance covered in cm/hour at 280 V towards the cathode (—) or the anode (+).

Buffer	*Molarity M*	*pH*	*Electrophoretic movement (distance covered from spot of application)*					
			DNP	*TPN*	*Nicotinamide*	*N′-methylnicotinamide*	*Nicotinic acid*	*Nicotinuric acid*
Acetate	0.1	4.8	—	—	— 0.6	— 4.6	+ 2.7	+ 2.7
Borate	0.033	8.9	+ 4.7	+ 5.5	— 1.8	— 9.0	+ 5.1	+ 3.7
Phosphate	0.033	7.0	—	—	— 1.5	— 6.8	+ 3.6	+ 2.4
Phosphate	0.033	10.2	—	—	— 1.0	— 6.9	+ 4.0	+ 2.8
Tris	0.05	9.2	—	—	— 1.7	— 8.3	+ 5.3	+ 4.6
Veronal	0.05	8.6	—	—	— 1.0	— 6.6	+ 3.8	+ 2.8

in the veronal and borate buffers tested, migrated in the direction of the anode. The resolution of the various compounds was found to be quite unsatisfactory when either acetate buffer or Tris buffer was used. Phosphate at pH 7.0 or 10.2, veronal pH 8.6 and borate pH 9.2 gave excellent separation of the various compounds. However, it was found that the spots, especially that of nicotinic acid, were somewhat diffuse when phosphate buffer was used, regardless of the pH. The use of veronal or borate gave not only good separation, but also very compact bands. A typical pattern of separation obtained with borate buffer is shown diagrammatically in Fig. 1.

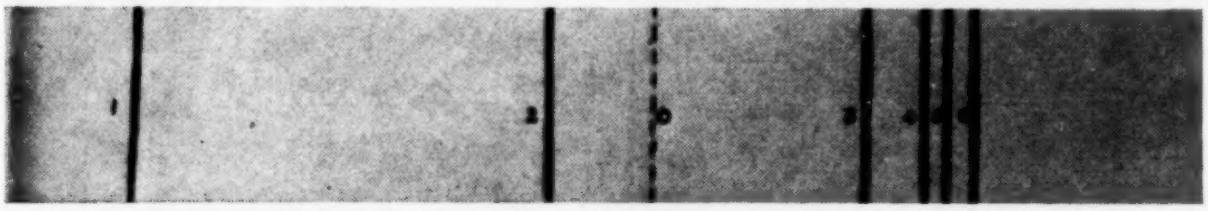

Fig. 1. Diagrammatic representation of the electrophoretic separation of nicotinic acid and its derivatives. Electrophoresis for 1 hour in $M/30$ borate buffer pH 8.9 at a difference of 280 V. 1, N′-Methylnicotinamide; 2, Nicotinamide; 3, Nicotinuric acid; 4, DPN; 5, Nicotinic acid; 6, TPN; 0, Position of application of sample.

As represented in Fig. 1, nicotinamide moves towards the cathode under the experimental conditions mentioned earlier. However, it was found that when the solution of nicotinamide was applied to the paper strip at its cathodic end, the nicotinamide band moved towards the anode, while there was no change in the electrophoretic behaviour of the other compounds. It is thus obvious that nicotinamide attains a position of mobility equilibrium somewhere between the cathodic end and the middle of the strip under the conditions of electrophoresis employed in these studies.

The foregoing studies have shown that the electrophoretic technique can be used for a quick and efficient separation of nicotinic acid derivatives. It was next considered necessary to find out the effectiveness of this technique for the separation of these compounds present in experimental samples. The nicotinamide deamidase system of *Corcyra* larva[2] was taken up for such an investigation. 1 ml of a 20% homogenate of larval tissue was incubated in a 2 ml system containing Tris buffer ($M/10$) pH 7.5 and 5 μmoles nicotinamide for 2 hours at 37°. At the end of the incubation, the reaction tube was heated in a boiling water bath for 10 minutes, and after centrifugation of the mixture, the almost clear supernatant was subjected to electrophoresis as detailed above, the sample being applied at the middle of the strip. The electrophoretogram was treated with cyanogen bromide and benzidine for the detection of bands corresponding to nicotinamide and nicotinic acid.

Earlier studies[3] have shown that the liver and the kidney of the rat are deficient in nicotinamide deamidase activity. In the present experiments a rat liver homogenate was incubated in the nicotinamide-containing reaction mixture mentioned above for detecting deamidase activity, and an electrophoretogram of the incubation mixture was developed as already described. The electrophoretic behaviour of incubation mixtures containing *Corcyra* tissue homogenate and rat liver homogenate is brought out in Figs. 2a and 2b respectively. It is seen that while there is a prominent band of nicotinic acid in the strip corresponding to the larval system, there is no detectable nicotinic acid in the strip on which the rat liver system has been subjected to electrophoresis. Since nicotinic acid in amounts as low as 0.25 μg can be detected by this method, these results conclusively demonstrate that rat liver, in contrast to *Corcyra* tissue, is completely devoid of nicotinamidase activity. This finding is in conformity with our earlier results obtained using microbiological assay procedures[3] and contradicts the report of PORCELLATI and co-workers[5] that rat liver homogenates carry out the deamidation of nicotinamide.

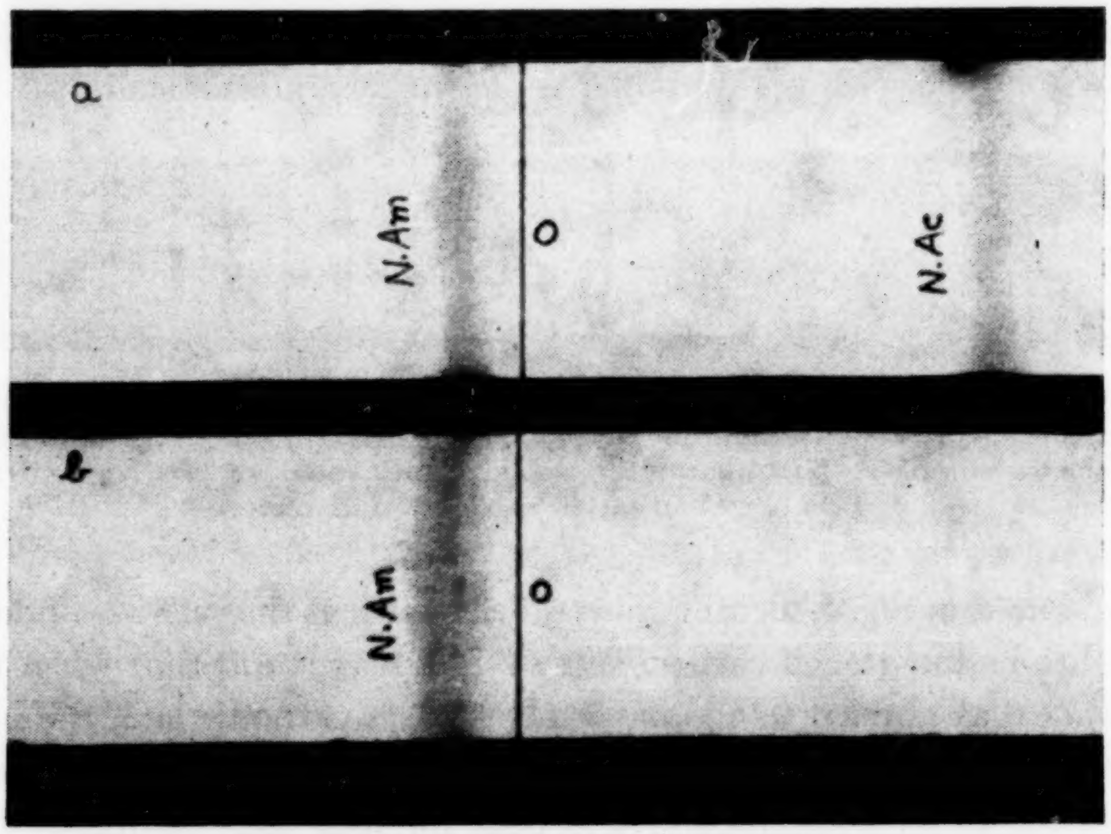

Fig. 2a, 2b. Electrophoretograms of nicotinamide deamidase incubation systems. 2a, *Corcyra* larval homogenate; 2b, Rat liver homogenate. o, Position of application of incubation mixture; N. Am = Nicotinamide; N. Ac = Nicotinic acid.

Recent studies on the biosynthesis of pyridine nucleotide coenzymes have brought to light the existence of two pathways for this biosynthesis, one starting with nicotinamide and the other with nicotinic acid. The tissues of the mouse have been shown to be capable of forming the pyridine nucleotides from nicotinamide as well as from nicotinic acid[6]. Similarly human erythrocytes contain enzyme systems mediating both the biosynthetic pathways[7,8]. Although the interconversion of nicotinic acid and nicotinamide in these systems is a distinct possibility, PREISS AND HANDLER[7] have demonstrated that human erythrocytes do not convert nicotinic acid to nicotinamide. In the present investigation, the capacity of human erythrocytes to effect the deamidation of nicotinamide has been examined; the erythrocytes of the rat and of the pigeon have also been similarly tested. The erythrocytes were collected from oxalated blood by centrifugation, washed with isotonic saline and finally suspended in this medium to give a 20% (v/v) suspension, which was tested for nicotinamide deamidase activity by the method described for *Corcyra* tissue and rat liver. None of the erythrocyte preparations exhibited the capacity for deamidating nicotinamide. It is thus apparent that the pathways of the biosynthesis of the pyridine nucleotides starting from nicotinic acid and from nicotinamide are independent of each other, the operation of either pathway being dependent on whether the vitamin is available in the acid or the amide form. However, it appears likely that in organisms wherein there is a pronounced tendency for the deamidation of nicotinamide this biosynthesis proceeds mainly from nicotinic acid. The deamidation of nicotinamide thus assumes significance not only as a pathway in the excretory metabolism of the vitamin but as a possible means for providing the nicotinic acid needed for the formation of the pyridine nucleotide coenzymes. Investigations on these lines are in progress.

There have been a few reports on the electrophoretic separation of nicotinamide derivatives, but invariably from the point of view of the biosynthesis and metabolism of the pyridine nucleotide coenzymes. Thus SILIPRANDI, SILIPRANDI AND LIS[9] have carried out the electrophoretic separation of nicotinamide, DPN and TPN, while more recently, KAPLAN and coworkers[10] and PREISS AND HANDLER[7] have employed this technique for the detection of the various intermediates in DPN synthesis. The present studies have been designed to bear mainly on the metabolism of nicotinic acid and nicotinamide; the electrophoretic technique described in this paper can be conveniently employed for quick and efficient separation of the various metabolites in studies on the excretory metabolism of the vitamin or in investigations of the deamidation or methylation of nicotinamide and the amidation or glycine conjugation of nicotinic acid. The technique has already been successfully adopted for the detection of nicotinamide deamidase activity in the tissues of several organisms.

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SUMMARY

The paper electrophoretic separation of nicotinic acid, nicotinamide, nicotinuric acid and N′-methylnicotinamide has been investigated.

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A DOUBLE-FRONTING PHENOMENON IN ONE-STEP DEVELOPMENT CHROMATOGRAPHY ON ANION EXCHANGERS

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INTRODUCTION

The development of chromatography during the last two decades has not only given us a very valuable tool both for purification and analysis of complicated mixtures of complicated substances, but has also revealed a great variety of pitfalls inherent in the methods. The main difficulties met with when using partition chromatography were studied by LESTER SMITH[1], who paid special attention to the possible mechanisms causing multiple zoning of a single solute. SCHROEDER[2] found in his work with adsorption chromatography on silicic acid that a homogeneous substance under certain conditions was split up into two zones and the same was shown by STRAIN[3] for pigments adsorbed on powdered sugar. Both authors came to the conclusion that this anomaly depended on precipitation and redissolving of the substance.

In adsorption and ion exchange chromatography of proteins it is necessary to work near the maximum capacity of the column and this may give rise to multiple zoning when the chromatogram is developed in several steps[4]. One-step development has been considered not to involve such risks. However, when chromatography on the anion exchanger diethylaminoethyl cellulose was used for the fractionation of a cobra venom[5], it was found that one-step development can give two distinct steps in the concentration of the effluent developing agent. Consequently, a homogeneous protein may be split up into two peaks even in one-step development. In the present work double-fronting of the developing agent on anion exchangers has been studied, and it has been shown that the phenomenon is caused by interaction with atmospheric carbon dioxide.

EXPERIMENTAL

Adsorbent

The adsorbents used were diethylaminoethyl cellulose (DEAE cellulose) according to PETERSON AND SOBER[6], triethylaminoethyl cellulose (TEAE cellulose) prepared as described by PORATH[7], and Dowex-2 with a cross-linking of 8–10%. The nitrogen contents of the DEAE and TEAE derivatives were 11.6 and 14.0 mg/g respectively, the determinations being based on the weight of vacuum-dried substance.

Before packing the columns the cellulose ion exchangers were thoroughly washed with 1 N hydrochloric acid, water and 1% sodium hydroxide. In the case of Dowex-2, the sodium hydroxide washing was omitted and the treatment with acid was continued until no more gas was evolved[4]. The adsorbent was suspended in about 20 times its volume of water and allowed to stand for 1–2 hours, after which the supernatant was decanted in order to remove the smallest particles. Then the suspension was deaerated and filled into the column under a water pressure of about twice the column length. A new column was always carried through a regeneration cycle before the first chromatogram.

Buffer system

This was tris(hydroxymethyl)aminomethane–hydrochloric acid (THAM–HCl), and the concentrations are given as normalities of HCl. In general the buffer was prepared by dissolving the appropriate amount of THAM in common distilled water, and then adding concentrated HCl until the desired pH was reached. After equilibration of the column with the starting buffer (checked by measuring the pH and the chloride concentration of the effluent) the chromatogram was developed in one or two steps with a buffer of the same pH but of higher ionic strength. The column was regenerated by washing with 1% NaOH, usually prepared from pellets, and then with 20–30 times the dead volume of the new starting buffer. Equilibration was never carried out directly from one buffer concentration to another but always over the regeneration cycle.

A fraction collector working on a time basis was employed, and in the figures the abscissa indicates tube number. The fraction volumes were about 1/8 of the dead volume.

The chloride analyses were made by the mercurimetric method[8], and concentrations are expressed in microequivalents per ml.

The total-carbonate concentration in the fractions was very roughly estimated in the following way: into each fraction 0.1 ml of 1% NaOH was pipetted, then 1.0 ml of the solution was added to 2.0 ml of concentrated barium chloride and the turbidity caused by the precipitate was immediately measured in a Klett-Summerson photometer. The concentrations are given in arbitrary units.

The concentration of ε-dinitrophenyl-lysine (ε-DNP-lysine) was determined as the extinction at 360 mμ using a Beckman B spectrophotometer and an 1-cm semi-micro cell. As a blank the developing buffer was used.

RESULTS

Figs. 1a and 1b show two chromatograms obtained on a 35 ml column (31 × 1.2 cm) of DEAE cellulose. In both cases the column had been equilibrated with 0.001 N THAM–HCl of pH 8.9, and the chromatograms were developed in one step with 0.076 N THAM–HCl of the same pH. The first experiment (Fig. 1a) was made without taking special precautions to prevent carbon dioxide from entering the system. In the other one (Fig. 1b) the buffer solutions had been prepared by first adding the hydrochloric acid to doubly distilled and air-equilibrated water and then deaerating the solution in a suction flask, after which the THAM was added. The sodium hydroxide used in the preceding regeneration cycle was prepared from commercially available concentrated solution (guaranteed to be carbonate-free) by dilution with water treated as above. Moreover, a drying tube filled with "Ascarite" (a sodium hydrate asbestos absorbent) was connected to the dropping funnel containing the influent solutions.

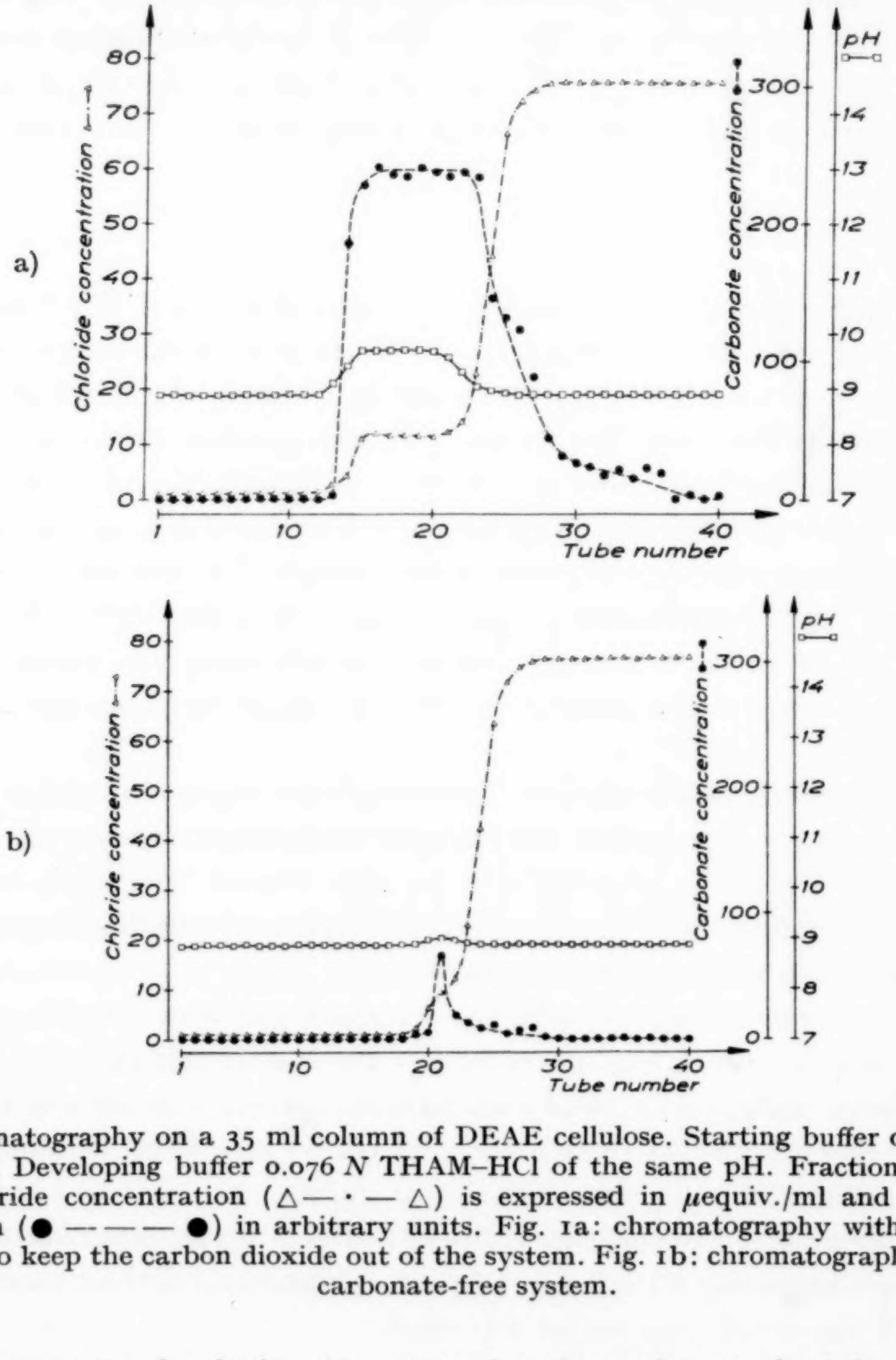

Fig. 1. Chromatography on a 35 ml column of DEAE cellulose. Starting buffer 0.001 N THAM–HCl, pH 8.9. Developing buffer 0.076 N THAM–HCl of the same pH. Fraction volumes about 1.8 ml. Chloride concentration (△— · —△) is expressed in µequiv./ml and total-carbonate concentration (● — — — ●) in arbitrary units. Fig. 1a: chromatography without any special precautions to keep the carbon dioxide out of the system. Fig. 1b: chromatography with a nearly carbonate-free system.

The chromatogram shown in Fig. 2 was obtained on the same column equilibrated with 0.001 N THAM–HCl of pH 8.9, but the development was here carried out in two steps, the first step with 0.023 N buffer and the second step with 0.075 N buffer. In this experiment and in those described below the sodium hydroxide solutions and the buffers had been prepared in the usual way. Some experimental data from chromatograms performed on DEAE cellulose at different pH values and with different starting and developing buffer concentrations are collected in Table I (see DISCUSSION).

Analogous experiments were also made on 19 ml columns of TEAE cellulose and Dowex-2. Fig. 3 shows a chromatogram on TEAE cellulose at pH 8.7. The starting buffer was 0.003 *N* THAM–HCl, and one-step development was performed with 0.078 *N* THAM–HCl of the same pH. No carbonate determinations were made. The results obtained on Dowex-2 were rather similar to those shown in Fig. 1a for chromatography on DEAE cellulose.

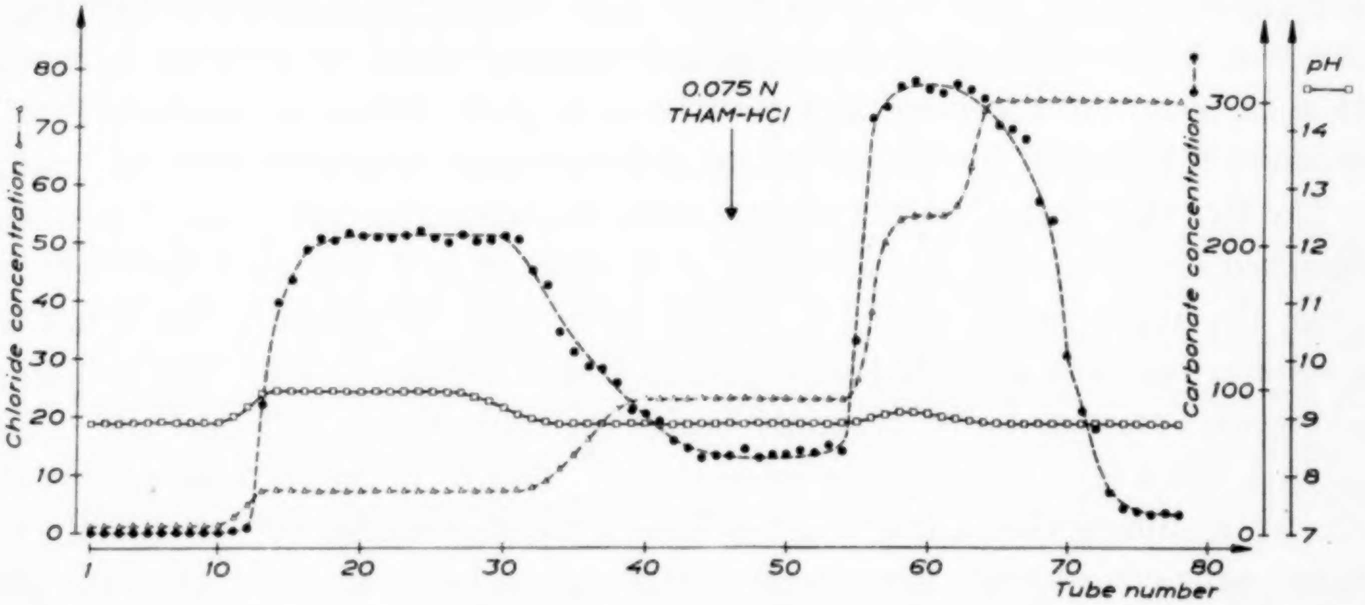

Fig. 2. Chromatography on a 35 ml column of DEAE cellulose. Starting buffer 0.001 *N* THAM–HCl, pH 8.9. Two-step development with 0.023 *N* THAM–HCl and 0.075 *N* THAM–HCl of the same pH. Fraction volumes about 1.8 ml. The start of development by the second agent at the top of the column is indicated by an arrow. Chloride concentration (△ — · — △) is expressed in µequiv./ml, and total-carbonate concentration (● — — — ●) in arbitrary units.

In order to simulate the conditions for protein chromatography, a 35 ml column of DEAE cellulose was eluted with ε-DNP-lysine dissolved in 0.023 *N* THAM–HCl of pH 8.9 (starting buffer) until the yellow zone had extended to about two thirds of the column length. The chromatogram was then developed in one step with 0.075 *N* buffer of the same pH, and the results are shown in Fig. 4.

DISCUSSION

If a column of an adsorbent having only one type of adsorbing site is in equilibrium with a solution of several adsorbable components (the starting solution), a change in the concentration of one of the components will upset all the existing equilibria and cause adsorption or desorption of the different components until new equilibria are attained. In the case studied here the adsorbable components have been the hydroxyl, chloride, carbonate and bicarbonate anions (the two latter species arising from the carbon dioxide taken up by the alkaline solutions), and with respect to these the eluting solution has differed from the starting solution only by an increased chloride concentration. Consequently hydroxyl, carbonate and bicarbonate ions would be displaced from the column during development, but as their concentrations are interdependent, they could not be expected to appear in the effluent as distinct zones. The bicarbonate released at pH 7.3 would tend to increase the pH, while the reverse would be true at pH 8.9 and no significant influence would be expected at an intermediate pH. A carbonate zone would give rise to an increased pH over the whole range. However, these changes as well as those caused by the displaced hydroxyl ions are counteracted by the buffer present in the solutions.

In Fig. 1a it can be seen that although one-step development has been used, the chloride front appears in the effluent in two well-defined steps. The anomalous step is accompanied by a large total-carbonate zone and by an increase in pH. Fig. 1b shows that it is possible to avoid this double-fronting of the chloride ion by working with a carbonate-free system, and thus there is good reason to conclude that the phenomenon is caused by the carbonate or bicarbonate displaced from the column.

The fact that under-nearly carbonate-free conditions the pH "hump" is strongly diminished indicates that it should not be considered as a displaced hydroxyl zone. During the regeneration with 1% sodium hydroxide (prepared in the usual way) the column becomes saturated with hydroxyl and carbonate ions. The subsequent washing with starting buffer certainly brings the column into hydroxyl and chloride equilibria, but a plausible explanation of the pH "hump" in the chromatogram can be given by assuming that a large part of the carbonate remains on the column, equilibrium at the considerably lower level of this ion not being attained. Then the developing buffer (at a pH of 7.3–8.9) will displace a large carbonate zone, most of which is gradually transformed into bicarbonate with a coincident liberation of hydroxyl ions. A starting buffer of a lower pH would be expected to elute more of the carbonate than one of a higher pH (the chloride concentrations being the same), and thus the amount of carbonate remaining would be smaller at the lower pH, which has in fact been found to apply.

In ideal displacement chromatography a mixture of different substances is resolved into a train of adjacent zones with only one substance in each zone[9]. The displacing agent must have an affinity factor which is higher than the corresponding value for any of the substances in the mixture, and the zones arrange themselves according to their affinity factors, the substance with the lowest affinity coming first in the train. As all the zones move at the same rate, the concentration of each zone is determined by the concentration of the displacing agent and the shape of the isotherm of the substance in question. Consequently the appearance of the anomalous chloride step would depend on the possibility for the chloride to assume different affinity values, *e.g.* under the influence of differences in pH.

In Table I the results from chromatograms performed under different conditions are collected. With the pH and the starting buffer concentration fixed, a developing buffer of higher concentration gives a higher and shorter anomalous chloride step, and also a higher and shorter pH "hump" (*cf.* Fig. 1a and Fig. 2, first part). When starting buffers of different concentrations are used, the pH being fixed, and elution is performed with the same developing buffer, a higher starting-buffer concentration gives a higher and shorter anomalous chloride step but a lower and shorter pH "hump"

TABLE I

EXPERIMENTAL DATA FROM CHROMATOGRAMS OBTAINED ON DEAE CELLULOSE AT DIFFERENT pH VALUES AND WITH DIFFERENT STARTING AND DEVELOPING BUFFER CONCENTRATIONS

pH of starting and developing buffers	*Chloride concentration of starting buffer Equiv./litre*	*Chloride concentration of developing buffer Equiv./litre*	*Chloride concentration in anomalous chloride step Equiv./litre*	*Height of pH "hump". pH units*
7.3	0.005	0.095	0.067	0.81
8.2	0.003	0.123	0.032	0.78
8.9	0.001	0.075	0.012	0.80
8.9	0.001	0.023	0.007	0.55
8.9	0.006	0.074	0.030	0.42
8.9	0.011	0.073	0.039	0.19
8.9	0.023	0.075	0.055	0.15

(*cf.* first and second parts of Fig. 2). This is easy to understand from the spreading of the isotherms at higher concentrations. The data in Table I can be used for plotting the quotient *hydroxyl ion concentration in anomalous step/hydroxyl ion concentration in starting buffer* against the quotient *chloride ion concentration in anomalous step/chloride ion concentration in starting buffer*. The relationship obtained is approximately linear, which is in good agreement with the hypothesis within the actual concentration ranges.

The reasoning above seems to imply that the affinity of the chloride ion and its pH dependence should by mutual adjustment be equal to the corresponding values for the carbonate-bicarbonate zone. However, it is possible that the application of the theory of ideal displacement to the actual situation is an oversimplification of the problem and that this should be attacked from another starting-point. A careful study of the effects responsible for the appearance of the anomalous chloride step would have to include frontal analysis of chloride as well as of total-carbonate at low concentrations and different pH's. These experiments must of course be made in a system protected from atmospheric carbon dioxide.

Two-step development may result in four steps in the effluent, as shown in Fig. 2. Total-carbonate data should not be used for any detailed interpretation, but it seems to be without doubt that the second total-carbonate peak really is higher than the first one. A corresponding proportion between the concomitant pH "humps" is not to be expected, as the buffer concentrations in the two cases are very different.

The chromatogram shown in Fig. 3 was obtained by one-step development on TEAE cellulose. Here the chloride front is divided into three distinct steps. The behaviour of the anomalous chloride steps under different conditions is qualitatively the same as on DEAE cellulose, and a possible explanation of the triple-fronting is that the ion exchanger used contained both DEAE and TEAE groups.

That a homogeneous compound can be split up into two zones by one-step development on DEAE cellulose is unambiguously shown in Fig. 4. In protein chromatography the corresponding amount of material adsorbed would be much smaller, and so a more complete separation of the zones would be favoured. As chromatographic heterogeneity of enzymes and hormones has recently been reported by many investigators (*cf.* [10–12]), the possibility exists that in some of these cases the underlying principle might be of the type described in this paper. It should be pointed out that the double-fronting phenomenon might well appear also in gradient development, especially as in many cases passage through the column will change the gradient to something more nearly resembling one-step elution. Chromatography with mixed buffers may also give rise to similar artifacts.

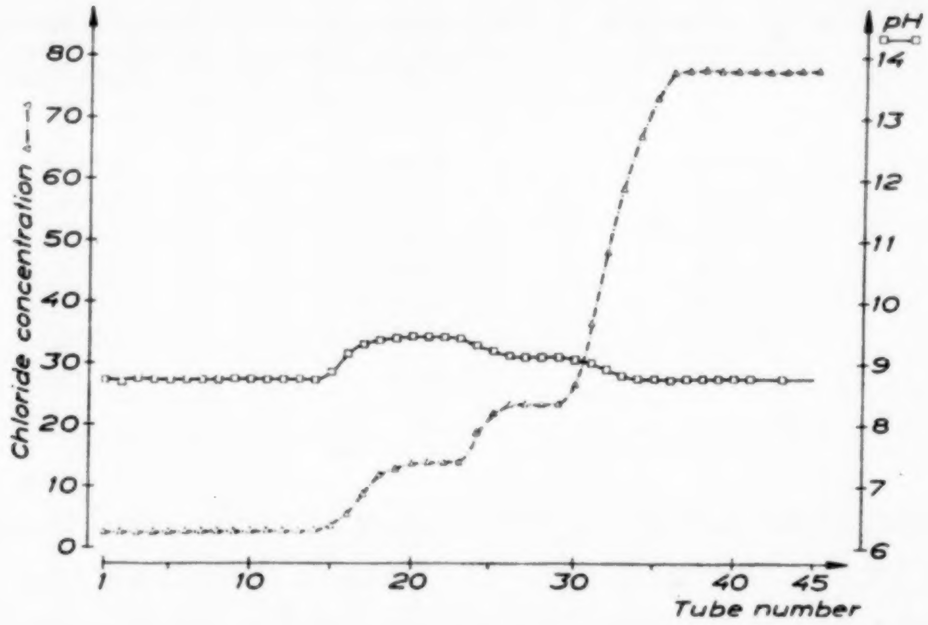

Fig. 3. Chromatography on a 19 ml column of TEAE cellulose. Starting buffer 0.003 *N* THAM–HCl, pH 8.7. Developing buffer 0.078 *N* THAM–HCl of the same pH. Fraction volumes about 1.0 ml. Chloride concentration (△ — · — △) is expressed in μequiv./ml.

For the interpretation of the chromatograms, determinations of pH and, if possible, of the concentration of the developing ion (or ions) should be made. By working with a carbonate-free system one can avoid the double-fronting, but in

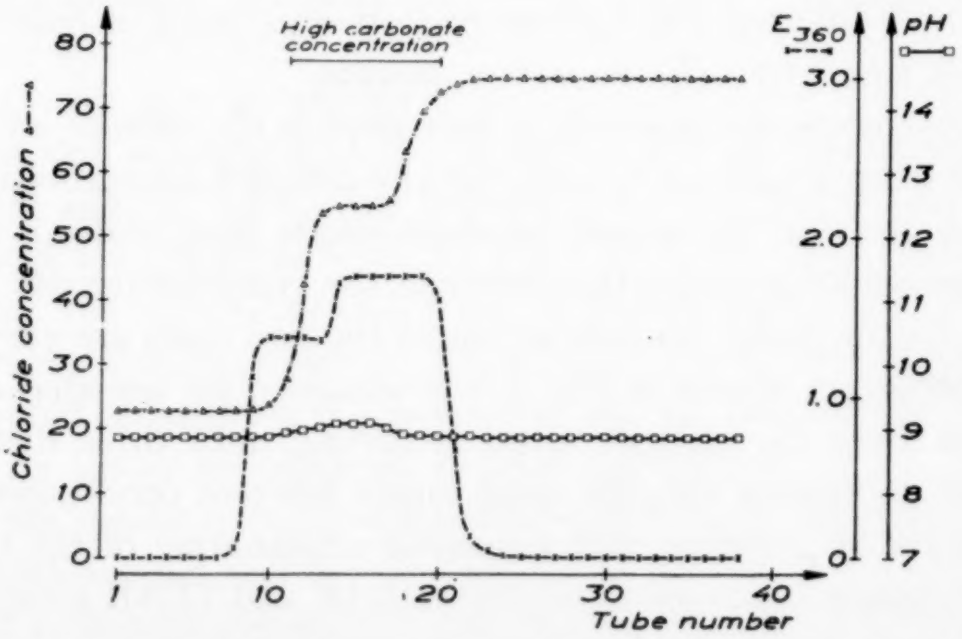

Fig. 4. Chromatography of ε-DNP-lysine on a 35 ml column of DEAE cellulose. The column saturated to about 2/3rds of its total capacity with ε-DNP-lysine dissolved in the starting buffer. Starting buffer 0.023 *N* THAM–HCl, pH 8.9. Developing buffer 0.075 *N* THAM–HCl of the same pH. Fraction volumes about 1.8 ml. Chloride concentration (△ — · — △) is expressed in μequiv./ml.

purification work it may often be quite harmless and sometimes even useful for obtaining better separations[5]. The important thing is to know its behaviour under the experimental conditions.

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SUMMARY

1. A study has been made of a double-fronting effect appearing in one-step development chromatography on anion exchangers.

2. It has been shown that the phenomenon is due to interaction with atmospheric carbon dioxide, and that it can be avoided by working with a carbonate-free system.

3. Possible mechanisms for the double-fronting have been discussed.

4. ε-DNP-lysine has been split up into two zones by one-step development on diethylaminoethyl cellulose (DEAE cellulose), thus showing the possibilities of artifacts caused by the double-fronting.

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IDENTIFICATION ET DOSAGE DES DROGUES DE LA FAMILLE DES PHÉNOTHIAZINES PAR CHROMATOGRAPHIE SUR PAPIER

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L'usage des dérivés de la phénothiazine, en particulier de la prométhazine (Phénergan) et de la chlorpromazine (Largactil), est de plus en plus répandu en médecine. Leur mode d'action peut les faire confondre avec les barbiturates ou les narcotiques. La méthode proposée découle d'une observation que nous avons faite en appliquant à des urines de patients la séparation chromatographique décrite récemment par nous[1] pour les alcaloïdes de la famille de l'opium. Les phénothiazines, qui se révèlent de la même façon que les opiacées, migrent cependant beaucoup plus rapidement et suivent même le front de la phase organique mobile (mélange d'isobutanol et d'acide acétique saturé d'eau). Un tel comportement nous a suggéré d'inverser les phases et d'utiliser plutôt comme phase mobile une solution aqueuse (sulfate d'ammonium à 5% saturé d'isobutanol). Les phénothiazines peuvent être ensuite facilement éluées et dosées grâce à une technique colorimétrique que nous avons décrite ailleurs[2].

Les phénothiazines étudiées dans le présent travail sont énumérées au Tableau I.

I. IDENTIFICATION

1. *Préparation des extraits*

Les phénothiazines peuvent être extraites presque quantitativement (86%) par un volume égal d'isobutanol à partir de leurs solutions alcalines. Il suffit d'alcaliniser l'urine avec quelques gouttes de soude à 10% et d'extraire directement.

2. *Papier*

Le Schleicher et Schuell No. 576 doit être préféré au Whatman No. 1 ou No. 4. Le papier ne subit pas de préparation particulière.

3. *Phases*

Une solution de sulfate d'ammonium à 5% dans l'eau distillée est agitée fortement avec un volume égal d'isobutanol. La solution aqueuse sert de phase mobile. L'excès d'alcool sert à saturer l'atmosphère de la chambre avant le développement.

4. *Solutions de référence*

Solutions de 100 μg des diverses phénothiazines par ml d'eau distillée.

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5. *Mode opératoire*

Les différentes étapes de la technique chromatographique (ascendante) sont les mêmes que décrites précédemment[1]. L'identification des zones se fait au moyen du même réactif (iodoplatinate de potassium tel que décrit d'abord par MUNIER[1, 3]).

6. *Résultats*

Les dérivés de la phénothiazine donnent une coloration bleue intense qui est beaucoup plus fugace que celle des alcaloïdes. On peut cependant révéler à nouveau les zones par une seconde application de l'indicateur. On peut facilement déceler 2 μg par cette méthode. Le Tableau I donne les valeurs de R_F calculées pour le front de la zone et un parcours constant de la phase mobile (soit, 22 centimètres du point d'application; temps: environ 7 heures à 22°).

TABLEAU I

VALEURS DE R_F DE DIVERSES PHÉNOTHIAZINES

Nom ordinaire	*Synonymes*	*Nom chimique*	*R_F (voir texte)**
Prométhazine	Phénergan, 3277 R.P.	(Diméthylamino-2′-propyl)-10 phénothiazine	0.71 ± 0.01
Levomépromazine	Nozinan, 7044 R.P.	Méthoxy-3-(diméthylamino-3′-propyl)-10 phénothiazine	0.67 ± 0.01
Promazine	Sparine (Wyeth)	(Diméthylamino-3′-propyl)-10 phénothiazine	0.62 ± 0.01
Chlorpromazine	Largactil, Thorazine, 4560 R.P. Aminazine	Chloro-3-(diméthylamino-3′-propyl)-10 phénothiazine	0.48 ± 0.01
—	Stelazine S.K.F.	Trifluorométhyl-3 (N-méthyl-pipérazimyl-4″-propyl-3′)-10 phénothiazine	0.23 ± 0.01

* Moyenne de 5 déterminations.

II. DOSAGE

1. *Réactifs*

Tampon citrate–phosphate (McIlvaine) à pH 4.0, dilué avec une partie égale d'eau.

Gomme ghatti, solution aqueuse à 2%.

Iodoplatinate de potassium (tel que décrit par MUNIER[3]).

2. *Mode opératoire*

Pour faire la chromatographie en vue d'un dosage, on applique deux aliquotes de l'échantillon en deux points voisins à la ligne d'origine. Après développement, on découpe le papier verticalement entre ces deux points. Une bande est révélée par l'iodoplatinate de façon à localiser la phénothiazine. La région correspondante sur la seconde bande est découpée, taillée en petits morceaux et agitée pendant 5 min en

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présence de 10 ml de la solution tampon. On ajoute 0.2 ml de la solution de gomme ghatti, on mélange, on ajoute 0.1 ml du réactif iodoplatinique. La coloration obtenue est lue à 610 mμ en présence d'un blanc des réactifs.

REMERCIEMENTS

Les auteurs tiennent à exprimer leurs remerciements à Melles L. VACHON et L. VERRAULT pour leur précieuse assistance technique.

RÉSUMÉ

Une méthode d'identification de quelques dérivés de la phénothiazine par chromatographie ascendante sur papier est décrite. La révélation des zones se fait par l'iodoplatinate de potassium. En vue d'un dosage, les zones peuvent être d'abord éluées et appréciées colorimétriquement par le même réactif.

SUMMARY

A method is described for the identification of some phenothiazine derivatives by ascending paper chromatography. The zones are revealed by means of potassium iodoplatinate. For quantative analysis the zones can first be eluted and then determined colorimetrically, using the same reagent.

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GAS–LIQUID CHROMATOGRAPHY: RETENTION VOLUMES OF THE METHYL ESTERS OF FATTY ACIDS WITH SPECIAL REFERENCE TO *n*-ODD-NUMBERED, *ISO* AND (+)-*ANTEISO* ACIDS

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In recent years, fats from a number of natural sources have been shown to contain small amounts of branched-chain fatty acids and *normal* odd-numbered fatty acids. Branched-chain fatty acids are present, for example, in the lipids of wool[1], bacteria[2], and plants[3, 4], and *normal* odd-numbered fatty acids have been found in hair lipids[5]. Branched-chain and *normal* odd-numbered fatty acids have also been found in body fats[6] and milk fats[7, 8, 9] of ruminants, in shark liver oil[10], and in the sebum of humans[11] and other animals[12]. In addition, the range of even-numbered saturated acids found in depot[13] and milk[14] fats of ruminants has been extended.

The isolation of the *normal* odd-numbered and branched-chain fatty acids, many of which are present in trace quantities only, has been accomplished by repeated low temperature crystallization from solvents and by fractional distillation. These procedures are extremely protracted and quantitative results are difficult to obtain.

With the development of gas–liquid chromatography it has been established that following the preparation of appropriate concentrates trace components can be quantitatively measured. Frequently, however, these acids have not been positively identified because of the lack of reference compounds. The purpose of this paper, therefore, is to provide further chromatographic data concerning the less common naturally occurring fatty acids, many of which have been isolated in this laboratory, so that in unknown mixtures these may be conclusively identified. The retention volumes of some of the acids used in the present investigation have already been reported by JAMES AND MARTIN[3] using Apiezon M and a heavy lubricating oil extract, and are repeated here because they are members of the series under investigation. The chromatographic data presented relate to three liquid phases, *viz.*, silicone grease, Apiezon M and polydiethylene glycol succinate. These phases are representative of the types most commonly used in the separation of esters of fatty acids by chromatography. The chromatographic data have been reported in the form recommended by DESTY[15].

EXPERIMENTAL

The gas–liquid chromatographic equipment used in this work has been previously described[16]. In all cases the column packing consisted of 1 part by weight of stationary liquid phase to 4 parts by weight of solid support (Celite 545, 48–85 mesh) which had been acid-washed and pre-treated with ethanolic NaOH[17]. The stationary liquid phase was dissolved in a solvent, added to the Celite, and the solvent removed by warming. During this operation the liquid phase was uniformly distributed either by stirring or by continuous rotation. Further, in the case of the polyester the columns were held at 200° for about 36 h in a stream of nitrogen, to remove volatile components[18]. Columns were packed by vibration of the column packing into straight tubes, which were subsequently bent in the shape of a U or coiled. The chromatographic columns were held at 200° or 220°.

Materials

Except where otherwise stated the esters used were prepared in this laboratory from either butterfat[8, 9, 14], ox fat[13, 19], mutton fat[20] or shark liver oil[10]. Other samples used were *normal* C_8, C_9, C_{10}, C_{12} saturated esters (Eastman Kodak, Rochester, N.Y.); *normal* C_{14}, C_{16}, C_{18} saturated esters (Unilever Ltd., Port Sunlight, Cheshire); methyl oleate, linoleate, linolenate, arachidonate (Hormel Institute, Univ. Minnesota), erucate (Light & Co., Slough) and elaidate (British Drug Houses Ltd.).

Chromatographic conditions for different column packings

Silicone grease (Dow Corning Corp.). Column length, 4 ft.; internal diam., 6 mm; weight liquid phase, 3.04 g; inlet pressure, 86.4 cm Hg; outlet pressure, 74.9 cm Hg; temp., 200° or 220°; corrected retention volume for n-C_{14}/g liquid phase, 231.8 ml; column resolution, 700 theoretical plates (for n-C_{14}).

Apiezon M (Shell Chemicals Ltd.). Column length, 4 ft.; internal diameter, 4 mm; weight liquid phase, 1.16 g; inlet pressure, 152.2 cm Hg; outlet pressure, 75.9 cm Hg; temp., 200° or 220°; corrected retention volume for n-C_{14}/g liquid phase, 1222.0 ml; column resolution, 1790 theoretical plates (for n-C_{14}).

Polydiethylene glycol succinate. Column length, 8 ft.; internal diam. 6 mm; weight liquid phase, 5.0 g; inlet pressure, 127.7 cm Hg; outlet pressure, 77.1 cm Hg; temp. 200° or 220°; corrected retention volume for n-C_{14}/g liquid phase, 58.4 ml; column resolution, 1870 theoretical plates (for n-C_{14}).

RESULTS

The carbon numbers of each homologous series when plotted against the logarithms of the retention volumes relative to methyl myristate fall on a straight line. These results agree with those obtained by JAMES AND MARTIN[3]. This, or similar graphic presentation of chromatographic data of esters of known composition, aids the prediction of the composition of unknown esters from their retention volumes and their carbon number. Positive identification of unknown mixtures, especially those containing *iso* and unsaturated esters is often simplified if they are run on more than one type of column.

The stationary phases Apiezon M and polydiethylene glycol succinate are particularly useful because of the different behaviour of the unsaturated compounds on the two columns (Table I).

TABLE I

RETENTION VOLUMES OF METHYL ESTERS OF FATTY ACIDS RELATIVE TO METHYL MYRISTATE AT 200° AND 220°

Methyl ester	*Silicone grease*		*Apiezon M*		*Polydiethylene glycol succinate*	
	200°	*220°*	*200°*	*220°*	*200°*	*220°*
n-Octanoate	0.11		0.07		0.17	
n-Nonanoate	0.16		0.10		0.24	
n-Decanoate	0.24		0.17		0.32	
n-Decenoate	—		—		0.41	
n-Undecanoate	0.33		0.27		0.42	
n-Dodecanoate	0.49		0.42		0.57	
11-Methyldodecanoate	—		0.56		0.66	
n-Tridecanoate	0.70		0.64		0.73	
12-Methyltridecanoate	—		0.85		0.88	
n-Tetradecanoate	1.00	1.00	1.00	1.00	1.00	1.00
13-Methyltetradecanoate	1.27		1.28		1.14	
(+)-12-Methyltetradecanoate	—		1.33		1.22	
n-Pentadecanoate	1.44		1.53		1.31	
14-Methylpentadecanoate	1.82		2.00		1.56	
cis-Δ^9-Hexadecenoate	—		2.15		2.08	
n-Hexadecanoate	2.06		2.38		1.77	1.70
15-Methylhexadecanoate	2.62		3.02		2.08	
Me ester of C_{19} acid with 3 Me side chains	—		3.10		1.60	
(+)-14-Methylhexadecanoate	—		3.17		2.16	
n-Heptadecanoate	2.91		3.47		2.29	
$\Delta^{9,12,15}$-Octadecatrienoate	—		4.41		5.60	
$\Delta^{9,12}$-Octadecadienoate	—		4.70		4.34	
16-Methylheptadecanoate	3.71		4.73		2.78	
trans-Δ^9-Octadecenoate	—		—		3.46	
cis-Δ^9-Octadecenoate	—		4.82		3.54	
Me ester of C_{20} acid with 3 Me side chains	—		4.91		5.46	
n-Octadecanoate	4.22	3.75	5.49	4.81	3.09	2.75
n-Nonadecanoate	6.11	5.17	8.65	7.24	4.02	3.50
n-Eicosanoate	8.48	7.14	—	10.40	5.50	4.59
n-Heneicosanoate	—	9.72	—	—	7.30	5.92
n-Docosanoate	—	13.60	—	—	9.83	7.91
$\Delta^{5,8,11,14}$-Eicosatetraenoate	—	—	—	—	9.62	—
Δ^{13}-Docosenoate	—	—	—	—	11.25	—
n-Tricosanoate	—	—	—	—	13.71	10.08
n-Tetracosanoate	—	—	—	—	17.51	15.12
n-Hexacosanoate	—	—	—	—	31.83	20.33

In the identification of unsaturated compounds it is important to know the effect, if any, of the position of double bonds on the retention volumes. Fig. 1 shows that the retention volume of methyl $\Delta^{5,8,11,14}$-eicosatetraenoate on the polyester phase is considerably lower than expected from the increase in retention volume with increased saturation in the C_{18} unsaturated series of esters. In the C_{20} tetraene the double bonds are nearer to the carboxyl group, and it appears that this shift has the effect

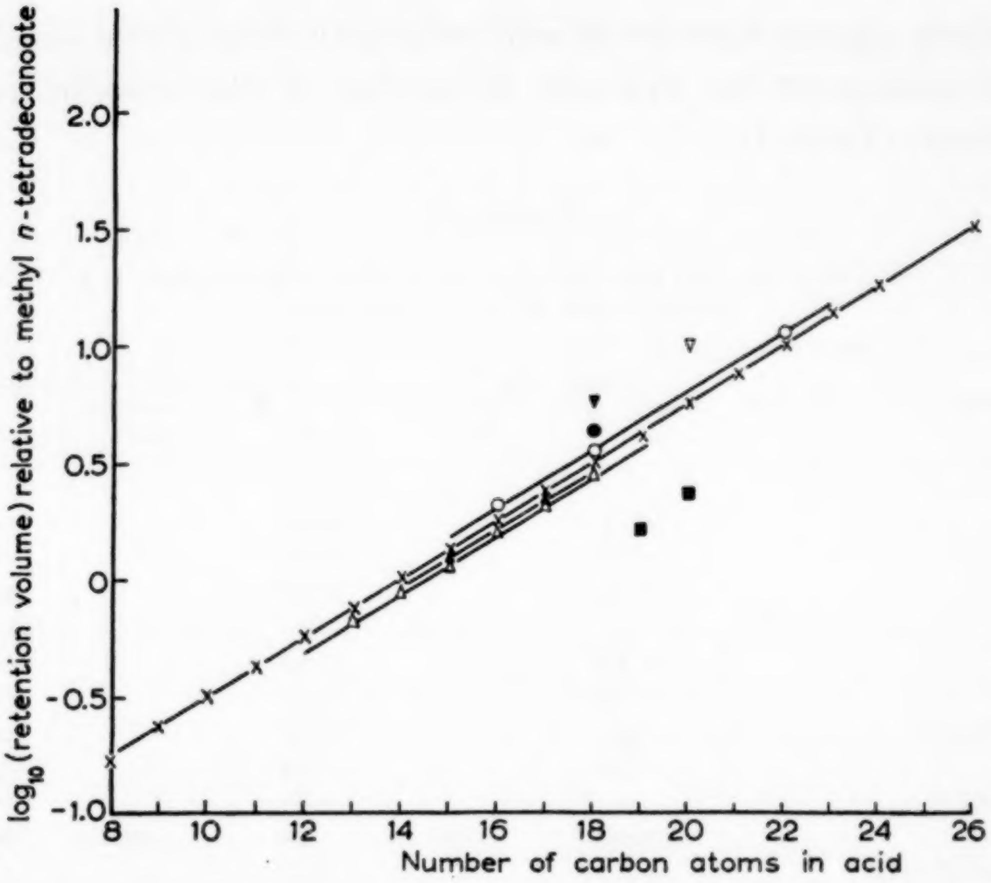

Fig. 1. The relationship between $\log_{10}$ of the retention volume relative to *n*-tetradecanoate and the number of carbon atoms in the molecule for the methyl esters of straight, branched and unsaturated acids from C_8 to C_{26}. ×, straight-chain acids; △, *iso* acids; ▲, (+)-*anteiso* acids; ■, multibranched acids; ○, monounsaturated acids; ●, diunsaturated acid; ▼, triunsaturated acid; ▽, tetraunsaturated acid.

of lowering the retention volume. This possibility will be tested when suitable esters become available.

ACKNOWLEDGEMENT

The authors are grateful to Dr. S. R. LIPSKY of the Yale University School of Medicine for the gift of the polydiethylene glycol succinate used in these experiments.

SUMMARY

Using gas–liquid chromatography with silicone grease, Apiezon M and polydiethylene glycol succinate as the stationary phases, the retention volumes of methyl esters of fatty acids have been determined at 200° and at 220°. The acids studied included *n*-odd and even-numbered saturated acids from C_8 to C_{26}, several unsaturated *n*-acids within the range of C_{18} to C_{22} and *iso*- and (+)-*anteiso* acids from C_{13} to C_{18} inclusive.

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DETERMINATION OF THE DEGREE OF UNSATURATION OF LONG CHAIN FATTY ACIDS BY GAS-LIQUID CHROMATOGRAPHY

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Studies of the fatty acid composition of natural fats, particularly those of animal origin, by gas–liquid chromatography have disclosed such a variety of saturated and unsaturated fatty acids (see JAMES AND MARTIN[1], JAMES AND LOVELOCK[2] and AHRENS *et al.*[3]) that it now seems unlikely that they can all be separated on one type of column stationary phase. Although the relative retention times of all the common acids in at least two stationary phases have been determined, peaks are frequently discovered that do not correspond in position with any of these acids. In this paper is described a method of using two chemically distinct types of stationary phase to define the degree of unsaturation and the chain length of such acids of unknown structure.

In the table of relative retention volumes recorded here, use is made of the shorthand designation of the fatty acids developed by Drs. DOLE AND AHRENS[3], of the Rockefeller Institute for Medical Research, New York. The cumbersome trivial or chemical names are replaced by a number describing the chain length, followed by a colon and a figure denoting the number of double bonds. Position of the double bonds is shown by a super-script and branching is indicated before the number defining the chain length, as is the configuration of the double bond. For example 18:0 is stearic acid, *cis* $18:1^9$ is oleic acid, *trans* $18:1^9$ is elaidic acid.

EXPERIMENTAL

Columns

All columns were of glass 4 ft. long and 4 to 5 mm I.D. The stationary phases were dissolved in a volatile solvent 40–60° light petroleum for Apiezon L grease (2.0 g to 8 g Celite) and chloroform for the poly-ethylene glycol adipate (2.5 g to 8 g Celite) mixed with the Celite (100–210 mesh), oven dried and packed by vibration. The Celite was pretreated with alcoholic alkali (JAMES, MARTIN AND HOWARD SMITH[4]), only when Apiezon L grease was used. Column efficiencies of 800 to 1,100 theoretical plates per foot of column were obtained.

Poly-ethylene glycol adipate was prepared by heating a mixture of 1.05 moles of ethylene glycol and 1.0 moles of adipic acid at 180°. When the mixture had melted, 200 mg of *p*-toluene-sulphonic acid was added to act as catalyst for the esterification. The mixture was heated for 2 h at 180° in a slow stream of nitrogen. At the end of this time the flask was evacuated on the water pump to remove any excess glycol and maintained at 180° *in vacuo* for a further hour. The resultant viscous liquid solidifies at room temperature and has a m.p. of 40–60°. The columns containing this stationary phase when operated at 180° have a useful life of 3–4 months.

Handling of the sample

After the usual procedures of saponification, extraction etc. the fatty acids were esterified either by refluxing with dry methanolic HCl (0.8 *N*) for 2 h or by treating with freshly distilled diazomethane at 0°. Where the total quantity of ester was of the order of 100 to 200 μg it was necessary to add a carrier liquid at this stage in order to increase the sample volume to 0.5 to 1.0 μl. The high sensitivity of the argon ionisation detector (LOVELOCK, JAMES AND PIPER[5]) allows the use of small volumes of diluted sample (see Figs. 1 and 2). Suitable carriers are 100–120° petroleum ether, chlorobenzene or methyl tri- or penta-decanoate. (These should be checked for purity on the gas chromatogram.)

Collection of fractions

Fractions were collected by attaching to the outlet of the detector, a wide tube loosely packed with defatted cotton wool wetted with methanol (JAMES AND MARTIN[6]). The material condensed in the tube was eluted with 10 ml of light petroleum to which was added 0.1 μl of a standard mixture of esters of the C_{10}, C_{12}, C_{14} and C_{15} acids (5% by weight) dissolved in either chlorobenzene or 100–120° petroleum ether. The solvent was evaporated in a small conical centrifuge tube wihch was repeatedly cooled so that condensed solvent washed the sample down to the bottom of the tube. The residual sample was taken up into a 3.0 μl micropipette and transferred to the top of the column. Retention volumes were measured from the centre of the air peak and relative retention volumes calculated with respect to methyl myristate.

DISCUSSION

Factors affecting the separation of long chain saturated and unsaturated fatty acids.

The relative position of the zones on a gas–liquid chromatogram is controlled by the differences in partial vapour pressure of the substances (solutes) when dissolved in the column stationary phase. The nature and magnitude of the cohesive forces between solute and solvent molecules defines the partial vapour pressure of the solute at constant temperature.

Non-polar solvents

When the solvent is a saturated paraffin hydrocarbon such as the Apiezon greases, the interaction forces between the fatty acid esters and solvent molecules are exclusively of the non-polar London dispersion type (provided that concentrations are kept sufficiently low so that solute–solute interactions cannot occur). These forces decrease with molecular weight and with branching of the hydrocarbon chain (for a discussion of these effects in hydrocarbons see JAMES AND MARTIN[6] and DESTY AND

WHYMAN[7]). Therefore, in this type of stationary phase unsaturated and branched chain acids move ahead of the corresponding saturated acids but di- and tri-unsaturated acids appear to be inseparable as do tetra- and penta-unsaturated acids. A list of relative retention volumes of acids of known structure is given in Table I.

TABLE I

RETENTION VOLUMES OF SOME FATTY ACID METHYL ESTERS OF KNOWN STRUCTURE RELATIVE TO METHYL MYRISTATE IN A NON-POLAR AND A POLAR STATIONARY PHASE

Acid	*Shorthand designation*	*Apiezon L at 197°*	*Poly-ethylene glycol adipate at 180°*
n-Pentanoic	5:0	0.019	—
n-Hexanoic	6:0	0.031	—
4-Methylhexanoic	*anti iso*-br- 7:0	0.041	—
n-Heptanoic	7:0	0.046	—
6-Methylheptanoic	*iso*-br- 8:0	0.057	—
n-Octanoic	8:0	0.071	0.141
6-Methyloctanoic	*anti iso*-br- 9:0	0.097	—
n-Nonanoic	9:0	0.11	0.193
8-Methylnonanoic	*iso*-br-10:0	0.144	—
n-Decanoic	10:0	0.173	0.264
8-Methyldecanoic	*anti iso*-br-11:0	0.235	—
n-Undecanoic	11:0	0.274	0.360
10-Methylundecanoic	*iso*-br-12:0	0.354	—
n-Dodecanoic	12:0	0.426	0.510
10-Methyldodecanoic	*anti iso*-br-13:0	0.567	—
n-Tridecanoic	13:0	0.580	0.710
cis-Δ^{9}-Tetradecenoic	*cis*-14:1^{9}	0.92	1.2
n-Tetradecanoic	14:0	1.0	1.0
12-Methyltetradecanoic	*anti iso*-br-15:0	1.35	—
n-Pentadecanoic	15:0	1.54	1.38
14-Methylpentadecanoic	*iso*-br-16:0	2.04	—
cis-Δ^{9}-Hexadecenoic	*cis*-16:1^{9}	2.09	2.26
trans-Δ^{9}-Hexadecenoic	*trans*-16:1^{9}	2.16	2.26
n-Hexadecanoic	16:0	2.34	1.96
14-Methylhexadecanoic	*anti iso*-br-17:0	3.24	—
n-Heptadecanoic	17:0	3.59	2.75
cis-$\Delta^{9,12}$-Octadecadienoic	*cis*-18:2^{9,12}	4.55	5.27
$\Delta^{9,12,15}$-Octadecatrienoic	18:3^{9,12,15}	4.60	6.88
cis-Δ^{9}-Octadecenoic	*cis*-18:1^{9}	4.75	4.33
trans-Δ^{9}-Octadecenoic	*trans*-18:1^{9}	4.95	4.33
cis-Δ^{6}-Octadecenoic	*cis*-18:1^{6}	4.87	4.35
cis-Δ^{4}-Octadecenoic	*cis*-18:1^{4}	4.95	4.36
trans-Δ^{4}-Octadecenoic	*trans*-18:1^{4}	5.15	—
n-Octadecanoic	18:0	5.5	3.86
$\Delta^{5,8,11,14}$-Eicostatetraenoic	20:4^{5,8,11,14}	8.25	12.85
Eicosapentaenoic	20:5	8.25	16.7
n-Eicosanoic	20:0	12.1	7.54
Docosahexaenoic	22:6	16.8	35.7
Δ^{13}-Docosaenoic	22:1^{13}	25.6	16.0
n-Docosanoic	22:0	26.3	—

The London dispersion forces are apparently affected not only by the number of double bonds but also by the position of the bond in the chain and by the configuration about the double bond (*cis*- or *trans*). For example, while methyl oleate (*cis*-$\Delta^{9:10}$-octadecenoate) has a relative retention time of 4.75, methyl elaidate (*trans*-$\Delta^{9:10}$-

octadecenoate) moves more slowly with a relative retention time of 4.95, methyl petroselinate (*cis*-$\Delta^{6:7}$-octadecenoate) has a value of 4.87 and *cis*-$\Delta^{4:5}$-octadecenoate of 4.95. These differences are sufficient to allow complete separation of most of these isomers, provided very high column efficiencies are attained. An example of the sort of resolution attainable with a 4,000 plate column is given in Fig. 1. A peak corresponding to positional and *trans*-isomers of oleic acid can clearly be seen.

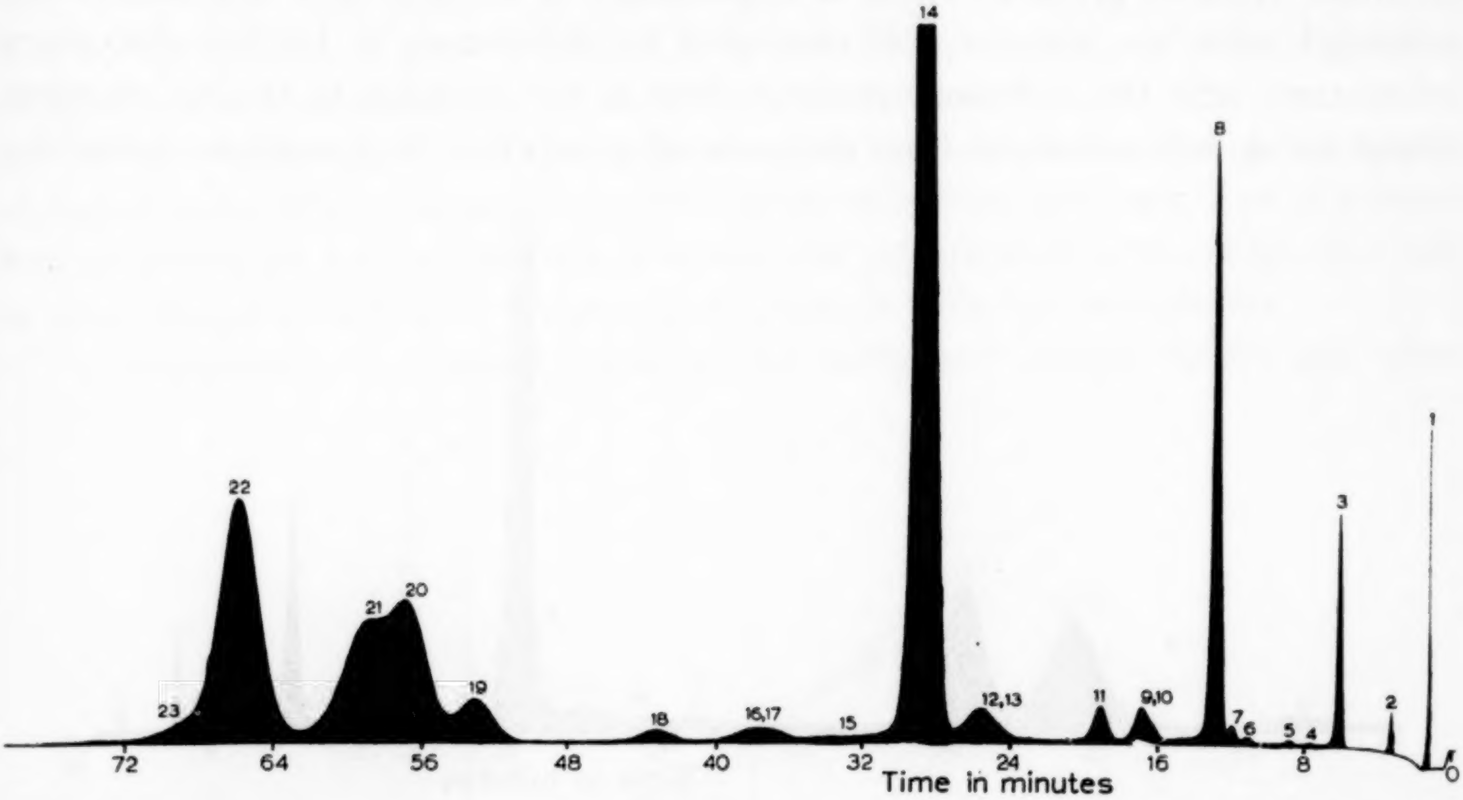

Fig. 1. The separation of 80 μg of fatty acids from human faecal lipids. Stationary phase: Apiezon L vacuum stopcock grease. Temperature: 197°. Argon pressure at inlet: 76 cm mercury above atmospheric pressure; at outlet: atmospheric pressure. Column length: 4 ft. straight tube. Detector: argon ionisation monitor, commercial version (Pye Scientific Instruments Ltd., Cambridge, England). Peak identification: 1. Solvent: light petroleum; 2. *n*-decanoic; 3. *n*-dodecanoic; 4. branched tridecanoic acids; 5. *n*-tridecanoic; 6. branched tetradecanoic; 7. monounsaturated tetradecanoic; 8. *n*-tetradecanoic; 9, 10. branched penta-decanoic acids; 11. *n*-penta-decanoic; 12. monounsaturated hexadecanoic; 13. branched hexadecanoic; 14. *n*-hexadecanoic; 15. highly branched heptadecanoic; 16, 17. branched heptadecanoic acids; 18. *n*-heptadecanoic; 19. linoleic; 20. oleic; 21. positional and configurational isomers of oleic; 22. stearic.

Polar solvents

In polar stationary phases such as poly-diethylene glycol adipate (Lipsky and Lansdowne[8]), poly-propylene glycol adipate (Orr and Callen[9]), and poly-ethylene glycol adipate (James[10]), specific intermolecular attraction occurs between the polarisable double bonds of the unsaturated acids and the ester groups of the polymer as well as non-polar London dispersion interactions. The magnitude of this polar effect increases with the number of double bonds as both Lipsky and Lansdowne[9] and Stoffel, Insull and Ahrens[11] have shown. This interaction retards specifically the unsaturated acids on the chromatogram and outweighs the London dispersion effect which exerts an opposing effect. However, the position of the double bonds and their steric configuration has no effect upon the retention volume of the monoenoic acids, so that such isomers are not resolved. This is demonstrated in Fig. 2. which shows separation of a similar mixture of acids to that shown in Fig. 1; only one peak is obtained for oleic acid and its isomers and in the tabulated figures of relative retention volumes given in Table I. Separations of straight and branched chain saturated acids are, however, still controlled by differences in London dispersion interactions with the stationary phase so long as the branches in the hydrocarbon chains are at such a distance from the carboxyl group that they exert no disturbing effect, *i.e.* branched chain acids still move ahead of the corresponding straight chain acids.

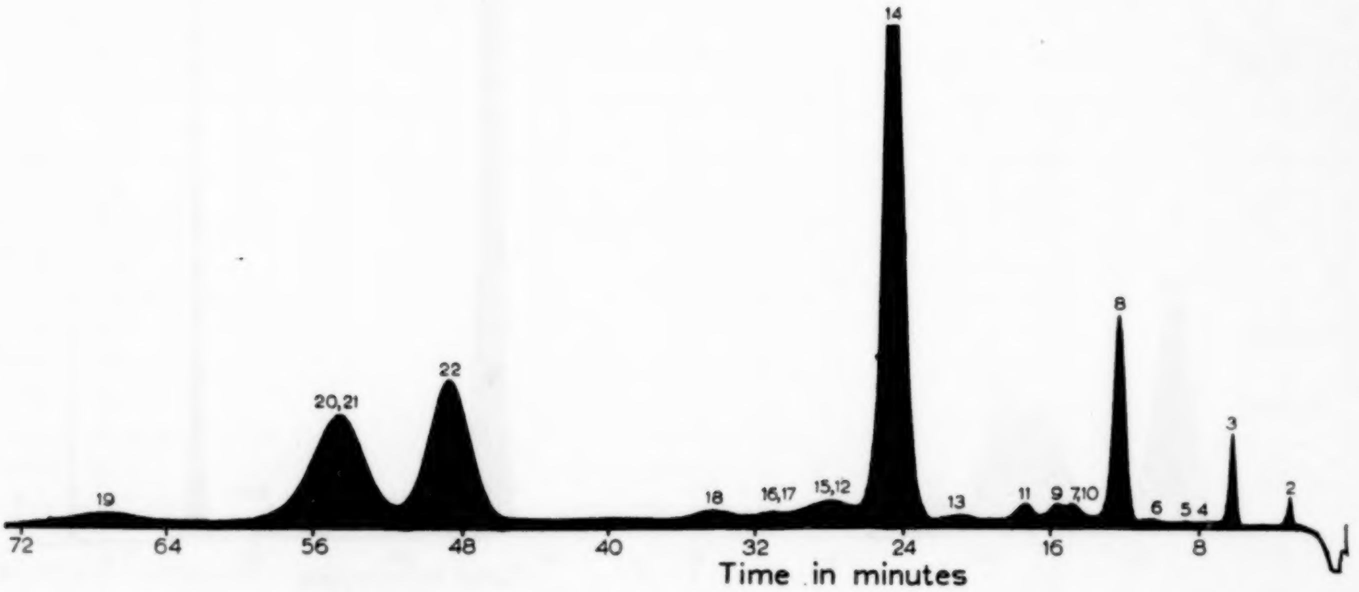

Fig. 2. The separation of 80 μg of a similar mixture to that shown in Fig. 1. Stationary phase: poly-ethylene glycol adipate. Temperature: 180°. Argon pressure at inlet: atmospheric + 57 cm mercury; at outlet: atmospheric pressure. Column length: 4 ft. straight. Detector: argon ionisation monitor, laboratory model. Peak identification: as in Fig. 1.

The London dispersion interactions between the esters and this polar stationary phase are smaller in magnitude than in hydrocarbon solvents so that retention volumes per g of stationary phase are smaller (Table II). Consequently the free energy of solvation of a $-CH_2-$ group is smaller, so that the $-CH_2-$ separation factor

$$\Delta CH_2 = \frac{\text{Retention volume of straight chain acid of } x \text{ carbon atoms}}{\text{Retention volume of straight chain of } x\text{—1 carbon atoms}}$$

is lower in the polyester solvent than in the hydrocarbon solvent, even at the lower temperature (Table II). In Fig. 3 is shown the relationship between $\log_{10}$ relative

TABLE II

RETENTION CHARACTERISTICS OF A NON-POLAR AND A POLAR STATIONARY PHASE

	Corrected retention volume of methyl myristate at column temperature per g of stationary phase (uncorrected for v.p. of water in flow meter) V_g°	*$-CH_2-$ separation factor*
Apiezon L at 197°	1320 ml	1.56
Poly-ethylene glycol adipate at 180°	507 ml	1.40

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retention volume and chain length of saturated fatty acids in the two stationary phases.

Determination of the degree of unsaturation of the long chain fatty acids

The complex array of peaks obtained with gas chromatograms of fatty acid esters obtained from natural fats can be identified in a number of ways:

1. By comparison of measured relative retention volumes with tabulated figures obtained under the same conditions with acids of known structure. This is a satisfactory procedure for the common saturated and unsaturated acids except that two or more components may be present in one peak on both types of column.

2. Comparison of chromatograms of the fatty acid sample before and after bromination shows which peaks are due to the unsaturated components since the polybromo acids are virtually non-volatile (JAMES AND MARTIN[1]).

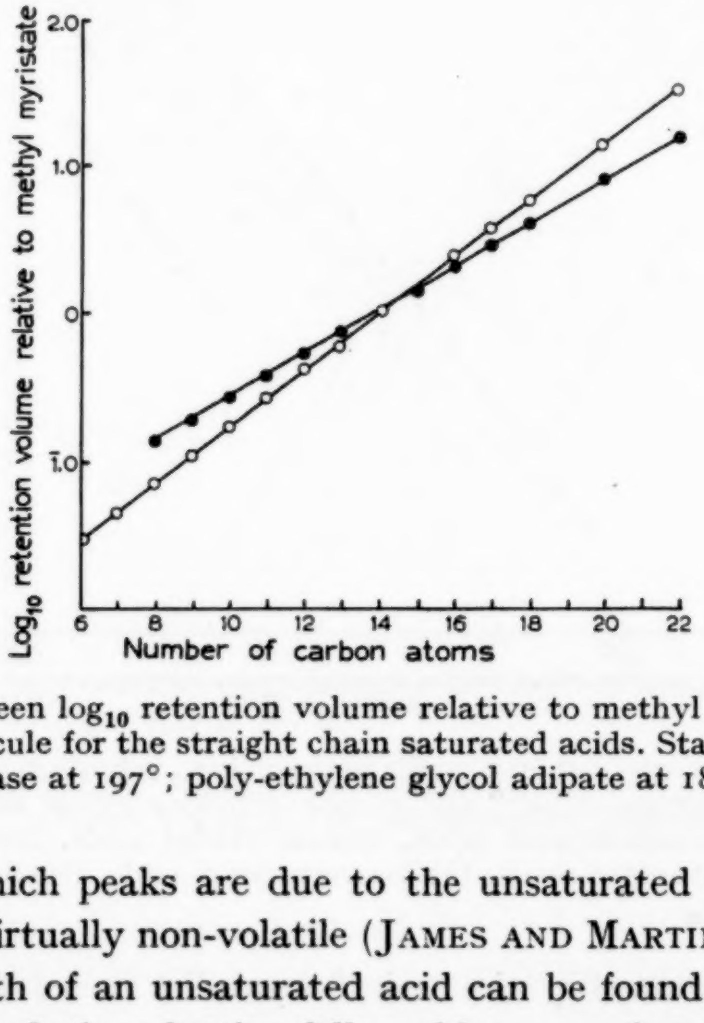

Fig. 3. Relationship between $\log_{10}$ retention volume relative to methyl myristate and number of carbon atoms in the molecule for the straight chain saturated acids. Stationary phase: Apiezon L grease at 197°; poly-ethylene glycol adipate at 180°.

3. The chain length of an unsaturated acid can be found by isolating the peak and subjecting it to catalytic reduction followed by rerunning the resulting saturated acid on the gas chromatogram (JAMES AND WEBB[12]).

4. The position of the double bonds in an acid can be defined by isolation of the fraction and then subjecting it to oxidative degradation either with potassium permanganate (JAMES AND WEBB[12]) or with ozone (STOFFEL AND AHRENS[13]). The fragments produced are then methylated and identified by the gas chromatogram. Such degradation procedures provide the most complete and unambiguous evidence of structures but for many analytical purposes it is sufficient to know the chain length and number of double bonds in the molecule.

5. The presence of polar or polarisable groups in a molecule can be detected by studying its retention time in two or more stationary phases showing different solute–solvent interactions. This was first demonstrated with aliphatic amines

(James[14]) using a non-polar hydrocarbon stationary phase and a poly-ether stationary phase that allowed donor-hydrogen bonding to occur. When relative retention volumes of a variety of amines in the two types of stationary phase were plotted against one another it was found that straight lines radiating from the origin could be drawn through the points corresponding to amines of the same type. Thus a single determination of relative retention volume in the two types of column under standard conditions defined an amine as primary, secondary or tertiary. A similar method of type determination in the hydrocarbon series was described by James and Martin[6].

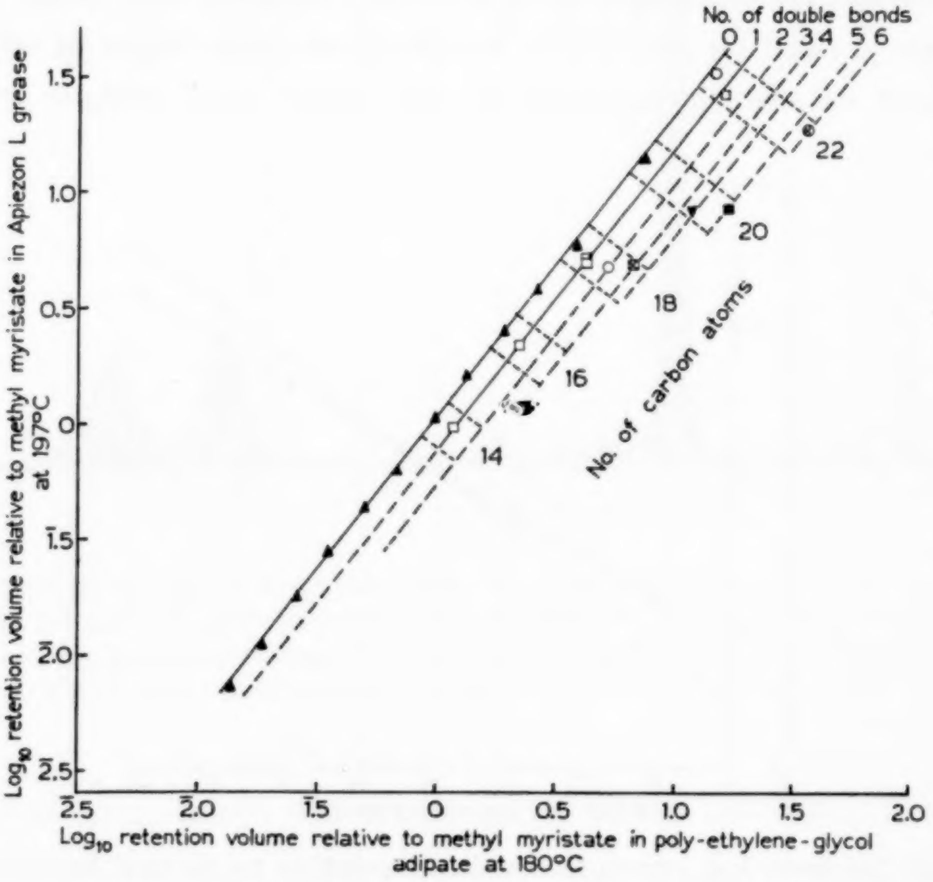

Fig. 4. Relationships for a variety of fatty acids between $\log_{10}$ retention volumes relative to methyl myristate in Apiezon L grease at 197° and in poly-ethylene glycol adipate at 180°. Saturated straight chain acids. Monounsaturated acids. Diunsaturated acids. Triunsaturated acids. Tetraunsaturated acids. Pentaunsaturated acids. Hexaunsaturated acids.

A simple graphical relationship of a series of parallel lines for the different types of hydrocarbon, was obtained by plotting the $\log_{10}$ relative retention volumes obtained in a polar and non-polar stationary phase.

Since the poly-ester stationary phases exhibit specific interactions with polarisable groups such as double bonds, it would be expected that $\log_{10}$ relative retention volumes for a variety of fatty acids in Apiezon L against the values obtained with the poly-ester should give points lying along a series of straight lines, each line corresponding to a given type of acid. In Fig. 4 is given such a graphical representation of the data in Table I.

It can be seen that the straight chain fatty acids fall along one line and the mono-unsaturated acids on a parallel line. Since only a few polyunsaturated acids of unambiguous structure were available, only single points were obtained for di-, tri-, tetra-, penta- and hexa-unsaturated acids, nevertheless, parallel lines were drawn through these points. A further test of this relationship was carried out by plotting the more extensive data published by STOFFEL, INSULL AND AHRENS[11] and AHRENS *et al.*[3]. These authors used Apiezon M grease at 197° and Reoplex 400 (poly-propylene glycol adipate) at 200°, and determined the structure of many acids by oxidative degradation. The result is shown in Fig. 5 and it can be seen that the di-, tri-, tetra-, and penta-unsaturated acids do lie along parallel lines, and the grid is similar in form to that given in Fig. 4. The dotted lines drawn at right angles show the spread of acids of identical chain length but varying degree of unsaturation. The spread is sufficiently small to prevent confusion between acids of odd and even chain length. These grids can therefore be used to define the number of double bonds in an acid by noting on which lines fall the $\log_{10}$ relative retention volumes obtained with the two stationary phases.

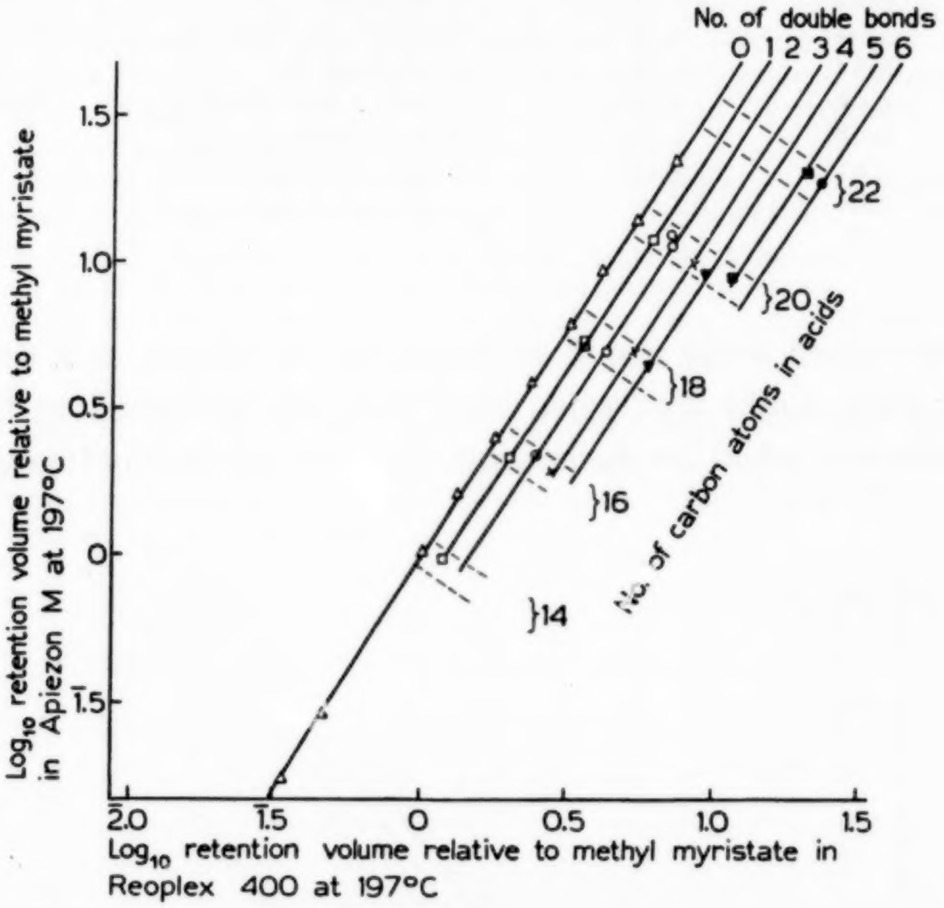

Fig. 5. Data plotted as in Fig. 4, having Apiezon M grease at 197° and poly-propylene glycol adipate (Reoplex 400) at 197° as stationary phases.

This procedure has been carried out by isolating peaks from an Apiezon chromatogram and rerunning them on the polyester chromatogram. The results are given in Table III and are expressed graphically in Fig. 6. The method is dependent on the absence of any degradation products in the isolated peaks. This has been unequivocally demonstrated by STOFFEL, INSULL AND AHRENS[11] using a number of both chemical and spectroscopic criteria. The structures assigned to the isolated acids are given in Table III and can be assumed with reasonable certainty.

It should be emphasized that the relationships demonstrated between structure and chromatographic behaviour apply only to the straight chain and simpler branched chain saturated acids. Aromatic acids, complex branched chain unsaturated and

TABLE III

IDENTIFICATION OF ISOLATED NATURALLY OCCURRING ACIDS FROM MEASURED RELATIVE RETENTION VOLUMES

	Retention volume in		*Identification from grid in Fig. 6*	*Shorthand designation*
	Apiezon L at 197°	*Poly-ethylene glycol adipate at 180°*		
A	0.92	1.20	monounsaturated C_{14}	14:1
B	1.30	1.19	branched C_{15}	br 15:0
C	1.35	1.27	branched C_{15}	br 15:0
D	1.98	1.77	branched C_{16}	br 16:0
E	3.02	2.24	highly? branched C_{17}	h br 17:0
F	3.14	2.55	branched C_{17}	br 17:0
G	4.26	2.76	highly? branched C_{18}	h br 18:0
H	0.68	1.77	triunsaturated C_{14}	14:3
I	3.11	3.17	monounsaturated C_{17}	17:1
J	8.66	11.7	triunsaturated C_{20}	20:3

conjugated unsaturated acids would be expected to behave in a different manner though there is little doubt that when more data are accumulated the structure of even these substances could be defined on the basis of chromatographic behaviour alone.

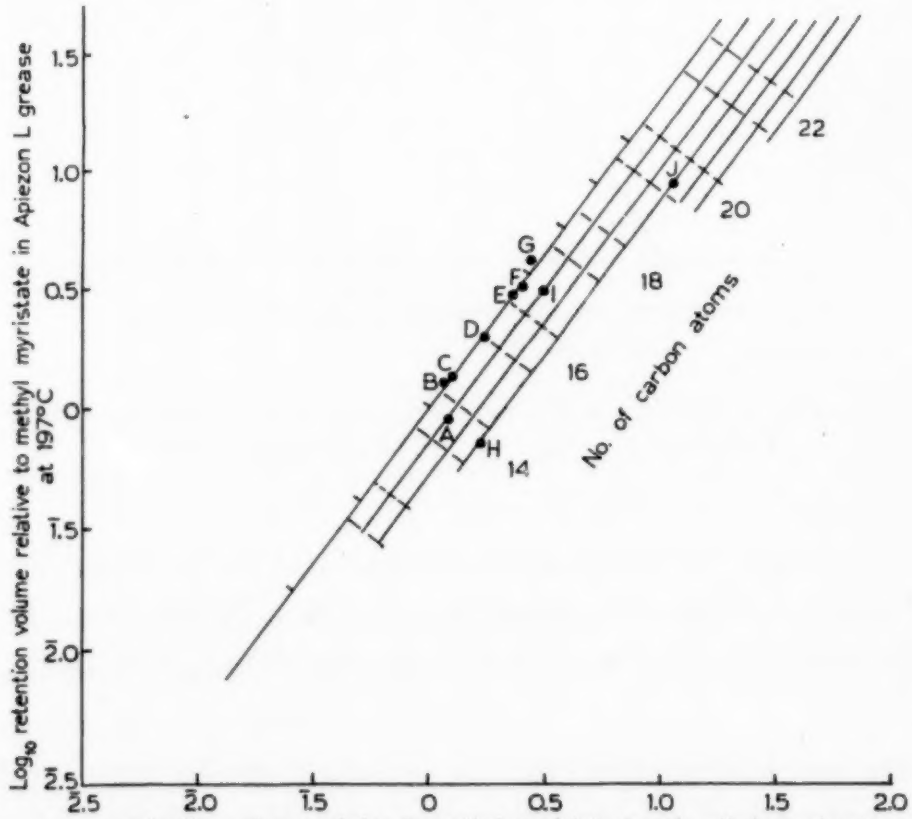

Fig. 6. Experimentally determined points from isolated peaks (see Table III) inserted on the grid from Fig. 4.

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I should like to express my thanks to Mr. H. Hadaway for such patient skilled experimental work and to Drs. Lundberg and Holman of the Hormel Institute for gifts of penta- and hexaenoic acids.

References p. 561.

SUMMARY

The number of double bonds and carbon atoms in an unknown fatty acid can be determined by comparison of its relative retention volumes on a polar and a non-polar stationary phase in a gas–liquid chromatogram using a grid established with known acids. Examples are given of acids isolated from animal fats.

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SHORT COMMUNICATIONS

Die gaschromatographische Trennung des Stickstofftrifluorides vom Kohlenstofftetrafluorid

Im Zuge unserer Arbeiten über die Elektrofluorierung von organischen Stickstoffverbindungen[1] ergab sich die Notwendigkeit einer sowohl qualitativen als auch quantitativen Analyse der anfallenden gasförmigen Elektrolysenprodukte. Auf Grund der ausgesprochenen Reaktionsträgheit gewisser zu erwartender gasförmiger Analysensubstanzen (vor allem NF_3, CF_4, N_2, O_2), die einer rein chemischen Untersuchung grosse Schwierigkeiten entgegensetzt, erschien in diesem Falle die Anwendung von gaschromatographischen Arbeitsmethoden als besonders angebracht.

In der vorliegenden Mitteilung sei nun im besonderen auf die gaschromatographische Trennung der beiden Substanzen NF_3 und CF_4 eingegangen, die sowohl durch G.S.C. (Gas–Solid Chromatography) wie auch durch G.L.C. (Gas–Liquid Chromatography) durchgeführt wurde und welche die bekannt schwierige physikalische Trennbarkeit dieser beiden Stoffe bestätigt. Aus theoretischen Überlegungen lässt sich ableiten, dass in diesem speziellen Falle eine Trennung viel leichter mit Hilfe der G.S.C., d.h. auf Grund der verschiedenen Siedepunkte (NF_3 Kp.—129°, CF_4 Kp. —128°)[2] als mit der G.L.C., d.h. auf Grund der unterschiedlichen Molekülstrukturen erreicht werden kann, trotzdem konnte gezeigt werden, dass sich prinzipiell beide Verfahren hierzu verwenden lassen.

Wie aus Tabelle I zu ersehen ist, konnte eine vollständige Trennung bei Verwendung einer 12 m langen, mit feuchtem Kieselgel beschickten Säule bei 0° und eine weitgehende Trennung mit einer 3 m langen, mit Aktivkohle beschickten Säule bei —21° erreicht werden. Es ist als sicher abzusehen, dass eine weitere Senkung der

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3	Kieselgel, feucht	0	9	beginnende Trennung
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3	Sterchamol — 22* + 20% Dimethylformamid	0	10	keine Trennung
3	Sterchamol + 20% Kel-F Polymer-Oil**	23	10	keine Trennung
3	Sterchamol + 20% Kerosen	23	10	keine Trennung
3	Aktivkohle	23	10	beginnende Trennung
3	Aktivkohle (siehe Fig. 2)	—21	45	weitgehendeTrennung

* Sterchamolwerke, Dortmund.
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Arbeitstemperatur im Falle der Aktivkohle ebenfalls eine vollständige Trennung ermöglicht hätte.

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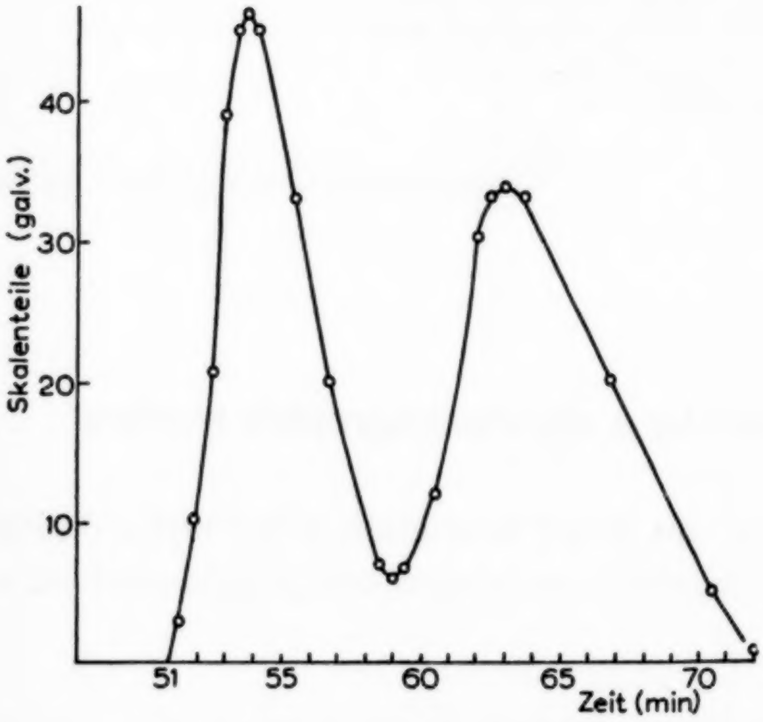
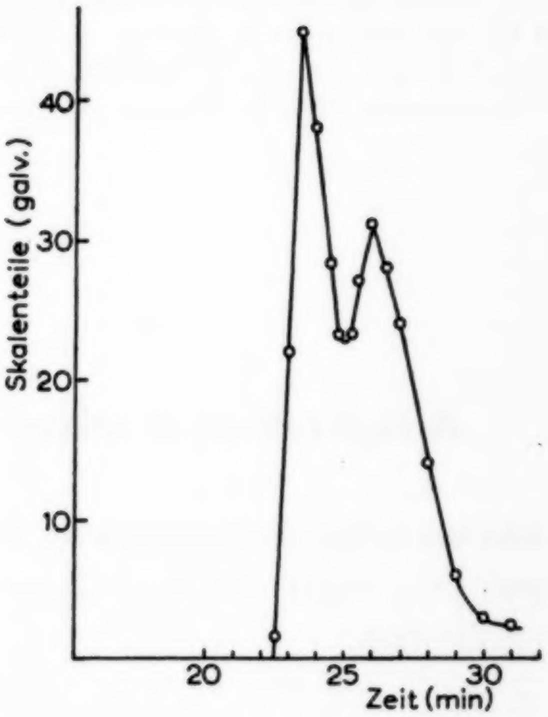


Fig. 1. Trennung eines NF_3–CF_4-Gemisches mit H_2 als Trägergas an Silicagel.



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Die Identifizierung der Chromatogramme erfolgte durch Versuche mit den jeweiligen Reinsubstanzen. Die quantitative Bestimmung der Einzelkomponenten in den Gasgemischen erfolgte nach der von CREMER UND HAUPT[4] ausgearbeiteten "Maxima-Methode", wonach die Höhen der Zackenmaxima bei vollkommen gleichen Versuchsbedingungen direkt proportional den aufgegebenen Substanzmengen sind. Nach Aufnahme einer Eichkurve für die jeweilig zu bestimmende Substanz lässt sich dann deren Anteil am Gemisch in einfacher Weise auf graphischem Wege ermitteln. Tabelle I gibt einen Überblick über die durchgeführten Versuche.

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Substanz	*Effektive Retentionsvolumina (l)*	
	An Aktivkohle — 21°	*An Kieselgel, feucht 0°*
NF_3	0.86	0.36
CF_4	0.95	0.45

SHORT COMMUNICATIONS

Die gaschromatographische Trennung des Stickstofftrifluorides vom Kohlenstofftetrafluorid

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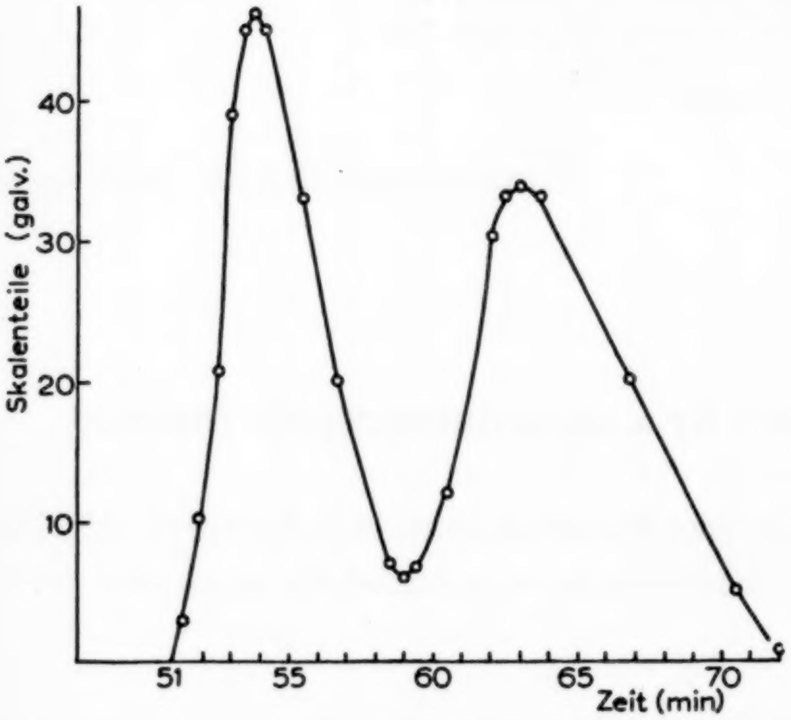

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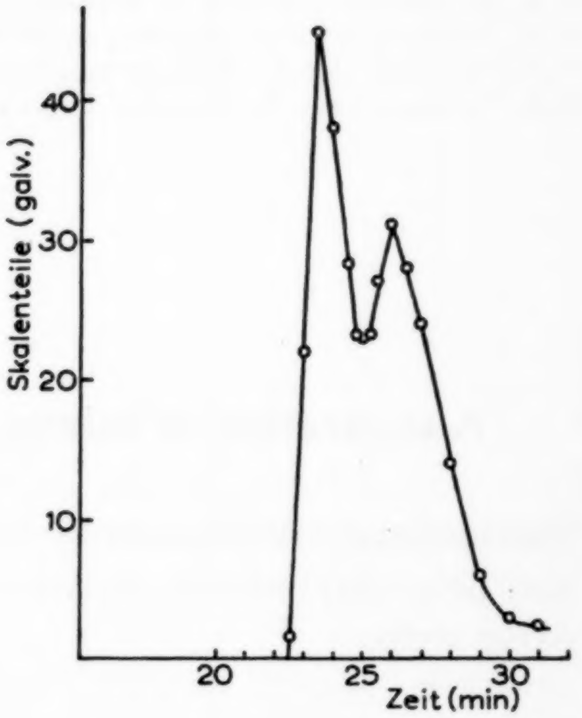

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Die aus unseren Ergebnissen berechneten, effektiven Retentionsvolumina für NF_3 und CF_4 bei den entsprechenden Temperaturen und für die geeigneteten Adsorbentien sind in Tabelle II angeführt.

Wir danken der Pennsalt Chemicals Corp., Philadelphia, Pa., U.S.A. für die freundliche Überlassung eines kleinen Druckzylinders mit NF_3.

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[1] A. Engelbrecht und E. Nachbaur, *Monatsh. Chem.*, 90 (1959) 371.
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Eingegangen den 23. April 1959

A separation of mixed polymers by a chromatographic method

The material investigated by this method was found to contain poly vinyl chloride and poly vinyl acetate polymers. These materials were successfully separated on a paper strip.

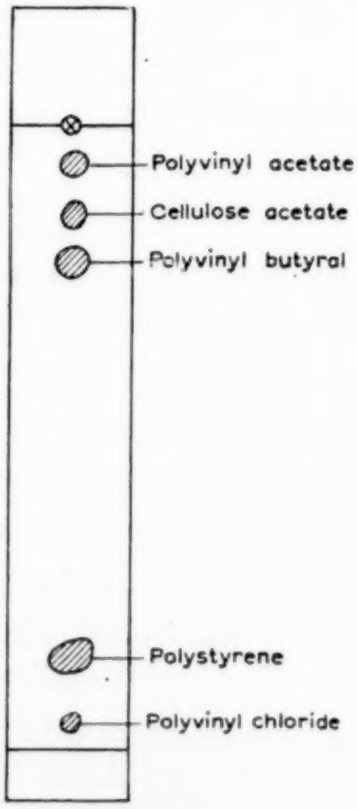

Fig. 1.

It seemed logical to assume that other polymers could also be separated by this means, and therefore, a mixture of the following polymers was prepared; poly vinyl chloride, poly vinyl acetate, polystyrene, cellulose acetate and poly vinyl butyral.

This was then spotted on to a Whatman No. 1 filter paper strip and run for 24 hours. It was then dried in air and sprayed with a mixture of 50 parts B.D.H. Universal Indicator and 50 parts distilled water.

A map of the spots is shown in Fig. 1. It will be seen that quite a good separation is obtained. However, in order to separate the first three spots even further a two-dimensional chromatogram was run.

The chromatogram was first developed with methyl isobutyl ketone. After drying at room temperature the chromatogram was developed in the second direction using methyl isobutyl ketone and methyl alcohol. After drying at room temperature the chromatogram was sprayed with B.D.H. Universal Indicator and the distilled water mixture. A map of these spots can be seen in Fig. 2.

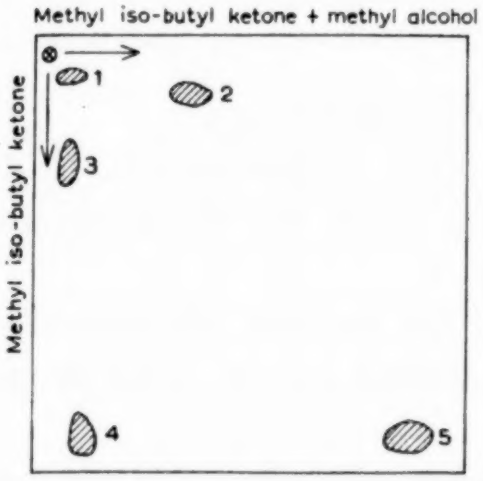

Fig. 2. 1. Poly vinyl acetate. 2. Poly vinyl butyral. 3. Cellulose acetate. 4. Polystyrene. 5. Poly vinyl chloride.

The fact that four of the five polymers used for this experiment are vinyl based polymers indicates that the method exhibits an extremely high order of selection, in that it is sensitive enough to discriminate between such closely chemically related polymers.

The experience gained and the evidence obtained from these experiments would lead one to assume that it may be possible to separate mechanical mixtures of other polymers. It has previously been illustrated that commercial polymers can be separated by the above means[1]. To date no attempt has been made to separate inorganically based polymers.

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[1] D. J. VAUGHAN, *Nature*, in the press.

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Detection of enzymes by paper chromatography

The preparation and identification of enzymes produced by microorganisms generally involves, very accurate techniques and special apparatus. The aim of this research was to obtain enzymes of just sufficient purity to enable their activity to be tested by paper chromatography, a technique which is widely used in all laboratories today. In this paper, the detection of aspartase, transaminase, glucose oxidase, maltase and amylase by paper chromatography is discussed.

1. *Aspartase*[1]

Microorganism. Escherichia coli RL 103.

Medium. Meat extract 0.3%, trypticase 1%, Na_2HPO_4 0.1%, KCl 0.5%. The pH is adjusted to 7.4–7.6 with NaOH and the medium is distributed in Roux flasks.

Incubation. At 37° for 48 h.

Preparation. After 48 h the bacteria are harvested by centrifugation and the bacterial mass is vigorously aerated for 1 h. A few drops of saline solution are added for storing in the cold.

Identification. The detection of aspartase is based on its action on fumaric acid whereby aspartic acid is formed, which may be identified by paper chromatography. 0.5 g of fumaric acid neutralized with NaOH, are mixed with 1 g of NH_4Cl in 20 ml of water. Then 20 ml of a phosphate buffer, pH 7.5, and 10 ml of the enzyme preparation are added. After expelling the air with a vacuum pump, the flask is incubated for 26 h at 37°. The cells are separated by centrifugation and 5 drops of the supernatant are placed on a Whatman No. 1 paper. The chromatogram is developed in butanol–acetic acid–water (4:1:4) at room temperature for 20 h, and then sprayed with a 0.1% ninhydrin solution[2]. A distinct spot corresponding to that of pure aspartic acid was observed, whereas no spot appeared in the enzyme preparation to which no fumaric acid had been added.

2. *Transaminase*[3]

Microorganism. Escherichia coli RL 103.

Medium (according to THORNE[4]). Nutrient broth with 0.3% yeast extract, 0.1% glucose and 0.5% glutamic acid. Initial pH: 7.5.

Incubation. At 37° for 18 h.

Preparation. After 18 h the medium is kept in the cold for 1 h and the cells are harvested by centrifugation. The bacterial mass is washed with 1/10th the initial volume of cold water, again separated by centrifugation and then dried over P_2O_5 in the cold. The cell powder may be kept in the cold in a sealed vial.

Identification. The detection is based on the action of transaminase on alanine and α-ketoglutaric acid in the presence of pyridoxal phosphate, whereby aspartic and pyruvic acids are formed.

500 μmoles of α-ketoglutaric acid are mixed with 50 μmoles of alanine, 100 μg of pyridoxal phosphate and 100 mg of dried cell powder in 10 ml of phosphate buffer, pH 8.2. This mixture is then incubated at 37° and the reaction interrupted after 2 h and 24 h respectively, with 10% trichloroacetic acid. The precipitate is separated by centrifugation and 3 drops of the supernatant are placed on a sheet of Whatman No. 1 filter paper and developed in butanol–acetic acid for 24 h at room temperature. After drying the paper is sprayed with a 0.1% solution of ninhydrin and heated at 80° for 10 min.

The results obtained were:

(a) for the solution of the cell powder: an unidentified spot.

(b) for the test without incubation: one spot corresponding to pure alanine and one corresponding to the cell powder.

(c) for the test incubated for 2 h: one spot corresponding to pure alanine, a second one corresponding to pure aspartic acid and a third one to the cell powder.

(d) for the test incubated for 24 h: the same results as in the previous test, but with greater intensity of the spot corresponding to aspartic acid.

3. *Glucose oxidase*[5,6]

Microorganism. Penicillium notatum ATCC 8251 grown as a surface culture in Roux flasks.

Medium. Glucose 4.0%, KCl 0.05%, KH_2PO_4 0.1%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MgSO_4 \cdot 7H_2O$ 0.05% and $NaNO_3$ 0.2%. Initial pH: 6.0–6.5.

Incubation. At 25° for 14 days.

Preparation. The culture medium is filtered, placed in the cold overnight and the pH adjusted to 3.5 with 10% phosphoric acid. Alcohol saturated with benzoic acid (50 ml/l of medium) is added and the precipitate separated by filtration. After washing with cold water saturated with benzoic acid, the precipitate is dissolved in cold acetone, using twice the volume of alcohol previously added.

Identification. The enzyme was detected by its specific action on glucose[7]. On a Whatman No. 1 filter paper, 2 drops of 1% glucose solution were spotted and dried. The chromatogram was run in butanol–acetic acid for 20 h at room temperature. After drying the paper was soaked in the solution of the enzyme and left to dry in the air for 15 min. Then it was treated with a freshly prepared solution of KI 5% and starch 1%. The gluconic acid formed by the enzymic reaction attacks the iodide liberating iodine which reacts with starch to give the characteristic dark blue colour.

4. *Maltase*[8]

Microorganism. Aspergillus niger ATCC 1015; 5 ml of a 24 h culture was used for each 200 ml of medium.

Medium. Maltose 2%, $NaNO_3$ 0.2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, and $FeSO_4 \cdot 7H_2O$ 0.001%. Initial pH: 6.5 adjusted with NaOH.

Incubation. At room temperature with agitation for 6 days.

Preparation. After 6 days the culture fluid, at pH 4.5–5.0, contains the enzyme.

Identification. The detection of maltase is based on its action on maltose, yielding glucose. 5 ml of a 1% solution of maltose were incubated with 5 ml of the enzyme solution at 28° for 24 h. For the identification of the glucose formed, 3 drops of the solution were placed on a Whatman No. 1 filter paper and developed in butanol–acetic acid for 20 h at room temperature. The glucose oxidase described above was used for the identification reaction.

5. *Amylase*[9]

Microorganism. Bacillus subtilis ATCC 9789, using 5 ml/l.

Medium. 5% rolled oats and 2% $CaCO_3$. Before sterilizing, the medium is left overnight in the cold to avoid foaming.

Incubation. At 37° for 10 days. TILDEN[9] incubated for 3 weeks, but 10 days proved to be sufficient for a qualitative reaction.

Preparation. The cells are separated by centrifugation and the supernatant is treated with twice its volume of cold acetone. The precipitate is filtered off on a Büchner funnel and redissolved in cold water (1/10th of the initial volume).

Identification. The amylase thus obtained was identified by a reaction used in paper chromatography[10], which is based on the action of amylase on starch. 5 drops of the enzyme solution are placed on Whatman No. 1 filter paper and dried. The chromatogram is developed in 50% acetone for 8 h in the cold. The paper is then placed on a dish containing 2% of agar-agar and 1% of starch. After incubating at 37° for 3 h the paper is removed and a solution of 0.01 *N* iodine is poured over the dish. The presence of amylase is indicated by a white spot on a dark blue background.

Acknowledgements

Grateful thanks are due to Dr. RAYMUNDO MONIZ DE ARAGÃO for advice and assistance during this study. The financial support of the National Research Centre of Brazil is also gratefully acknowledged.

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[1] J. H. QUASTEL AND B. WOOLF, *Biochem. J.*, 22 (1928) 474.
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Received April 10th, 1959

Studies on the thyrotropic substances by papergraphy*

In all but a few[6–8] of the numerous methods for the purification of the thyrotropic hormone reported in the past few years chemical procedures were used.

Recently, however, paper electrophoresis, especially, continuous paper electrophoresis, has been employed for the purification of this hormone in our laboratories. This appears to be a more convenient and rapid procedure than ordinary chemical methods.

The separate components obtained from the thyrotropic hormone by the abovementioned methods were examined for thyrotropic and gonadotropic activity. The protein associated with the thyrotropic hormone in rat serum was also separated and examined.

Paper chromatography was used to identify the carbohydrates in the thyrotropic hormone, these has been briefly described by FRAENKEL-CONRAT *et al.*[9] in 1940 and their presence confirmed by STEELMAN *et al.*[6] and FELS *et al.*[7], but only by means of a positive reaction in the Molisch test.

Methods and results

The thyrotropic hormone used was Pretiron a preparation of Schering A.G., Berlin. It was separated into its components by paper electrophoresis and continuous paper electrophoresis.

1. *Paper electrophoresis*

(a) Horizontal paper electrophoresis was conducted in barbital (veronal) buffer, pH 8–8.6, ionic strength $\mu = 0.05$. The current was about 20 mA/10 cm, the voltage 250 V/28 cm and the duration 18 hours. After separation, the components of Pretiron (0.005 g) were stained with Amino Black 10B; at pH 8–8.5 at least three fractions were found. Parallel fractions on the same papers that had not been stained were cut out and eluted with 0.05 ml of physiological solution. The aliquots were assayed by GREENSPAN's method[11] and the order of the thyrotropic activities was found to be as follows:

$$\text{Fraction II} > \text{Fraction I} > \text{Fraction III} = 0$$

The fractions I, II and III were numbered from the positive electrode. At pH 5–7, in NaCl–NaAcO–HAcO and NaCl–Na_2HPO_4–$NaHPO_4$ buffers[10], two fractions were separated, but, the thyrotropic activities of these fractions were not so high as at pH 8–8.6.

(b) Continuous electrophoresis was carried out in barbital buffer and phosphate buffer at pH 8–8.6, ionic strength $\mu = 0.05$, under the following conditions: current

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* The term papergraphy embraces all the analytical methods in which filter paper is used, *e.g.* spot tests with filter paper, paper chromatography, and paper electrophoresis, etc. This general term has been suggested and used by MORI *et al.* since 1952[1–5].

17 mA/30 cm, voltage 540 V/45 cm, duration 48 hours. The aliquots were assayed for thyrotropic activity and the results obtained are shown in Fig. 1.

Contamination of the above mentioned fractions by gonadotropic substances was assayed by the mice-uterus-weight methods[12]. The highest activity was found in the band II.

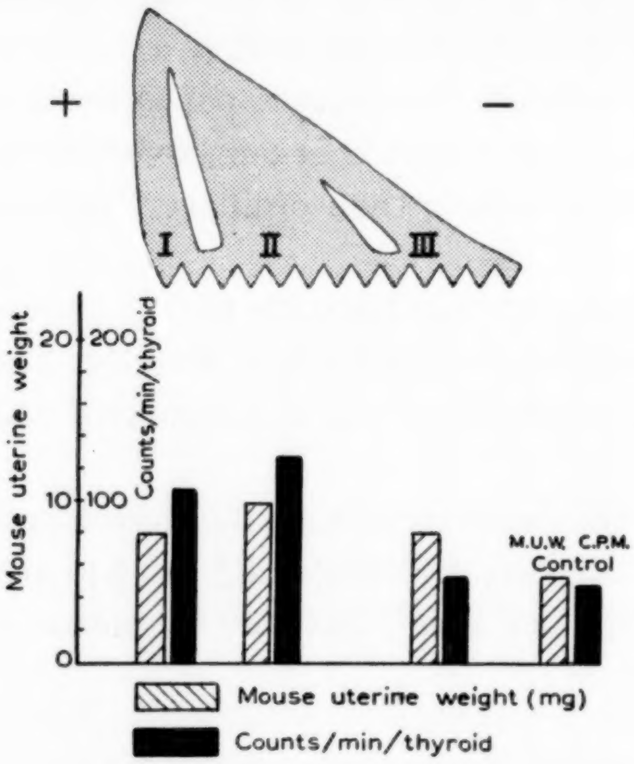

Fig. 1. Complete separation of components of Pretiron by continuous paper electrophoresis.

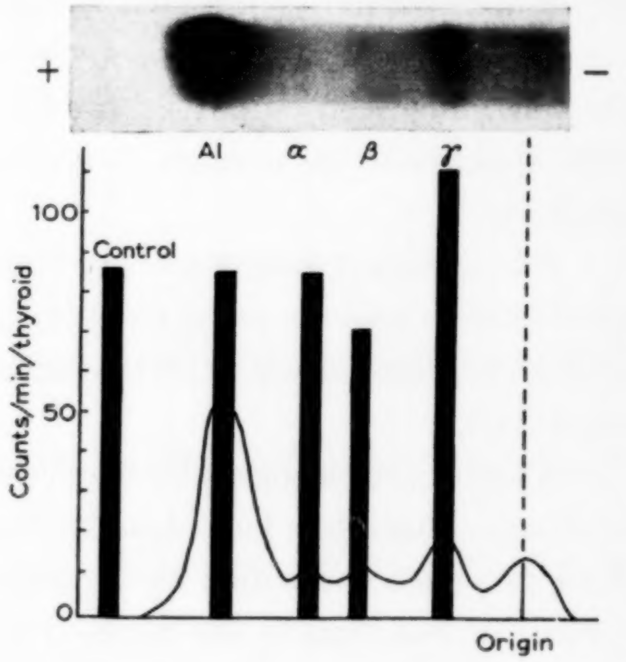

Fig. 2. The protein associated with thyrotropic hormone in rat serum.

(c) The serum protein of rats, which had been sacrificed 2 hours after the injection of Pretiron, was separated into four fractions by paper electrophoresis. These were assayed by GREENSPAN'S method and it was confirmed that the thyrotropic activity was always associated with and transported by the γ-globulin in rat serum, as shown in Fig. 2.

2. *Paper chromatography*

Samples: Pretiron (18 mg) and the components I, II and III that had been separated from 18 mg of Pretiron by continuous electrophoresis, were hydrolysed with 1 ml N HCl in a closed tube at 100° (oil-bath) for 2 hours.

Reagents: *Aniline hydrogen phthalate*. This was prepared by the methods of PARTRIDGE[13], by adding aniline (0.93 g) and phthalic acid (1.66) g) to water-saturated butanol (100 ml).

Naphthoresorcinol[14]. 0.2% (w/v) naphthoresorcinol in acetone solution are mixed with 3 N phosphoric acid, 5:1 (v/v) before use. The chromatograms are dipped in this solution for 1 min and then allowed to dry at room temperature before being introduced into the oven at 100–105° for 5–10 min. Under the conditions used this reaction is very selective for ketoses.

Silver nitrate–acetone solution. In our laboratories the ammoniacal silver nitrate solution of PARTRIDGE[15] and the alkaline silver nitrate solution of BURTON[16], were improved as follows:

Solution (a): silver nitrate (0.7 g) is dissolved in an aliquot of distilled water and made up to 100 ml with acetone. (b) 5 % NaOH solution. (c) 15 % aqueous $Na_2S_2O_3$ solution. The chromatograms are dipped into solution (a) and allowed to dry partially at room temperature and then left in solution (b) until the color of the reduced spot has developed. Then the chromatograms should be immediately taken out of the solution and washed with solution (c) to remove the background color of the paper. This procedure gives better results than ammoniacal and alkaline silver solutions.

Procedures. The used sugars as standards, xylose, fructose, sorbose, mannose, galactose, glucose, glucuronic acid and glucosamine, were dissolved in *N* HCl and spotted parallel with the samples on Toyo filter paper (No. 51). The filter papers were developed with the solvents *n*-butanol–pyridine–water (6:4:3, v/v), *n*-butanol–glacial acetic acid–water (4:1:1, v/v) or water-saturated phenol, for a minimum of 18 hours. The differences in the color of the chromatograms when developed with aniline hydrogen phthalate, naphthoresorcinol or silver nitrate–acetone solution, confirmed the presence of aldo-hexose, uronic acid and glucosamine in Pretiron. From the differences in the R_F values obtained in different solvents, it was concluded that mannose and galactose were present in the aldo-hexose. Component II from Pretiron gave three spots corresponding to galactose, glucosamine and uronic acid. The other components (I and III) gave two and one clear spots respectively, which developed colors with aniline hydrogen phthalate but not with naphthorssorcinol. It was confirmed that the spot from component III, which had no thyrotropic activity, was glucosamine.

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A simple arrangement, using a standard Geiger-Müller tube, for the continuous monitoring of radioactive effluents from a chromatography column

Several detectors have been described for measuring radioactivity in chromatographic effluents[1, 2, 3]; these are expensive and more complicated to operate than the one described below which incorporates a standard G.M. tube in an assembly that can be made in a short time. The one described has been in use in this laboratory for 6 months for detecting ^{14}C-labelled alcohols eluted with organic solvents from adsorption chromatography columns. It will satisfactorily detect low energy β emitting isotopes, *i.e.* ^{14}C (0.15 MeV) at levels as low as 0.0025 μC/ml in organic solution.

Construction. The G.M. tube used was a halogen-quenched type Mullard MX123 with a window thickness of 1.5 mg/cm^2. The body of the chamber and the clamping ring were made from 2 in. diameter brass rod; and the gasket from 1/16 in. polythene sheet. The inlet and outlet tubes were 3/16 in. internal diameter brass tubes soft soldered in position. The dimensions and constructional details are apparent from Fig. 1. Polythene tubing was used to connect the detector directly to the column and collector. The length and diameter of these connections was kept as small as possible to reduce the dead volume to a minimum. The detector was connected to a ratemeter (standard A.E.R.E. type 1037C) and recorder. The volume of the cell including inlet and outlet tubes is about 1.8 cc.

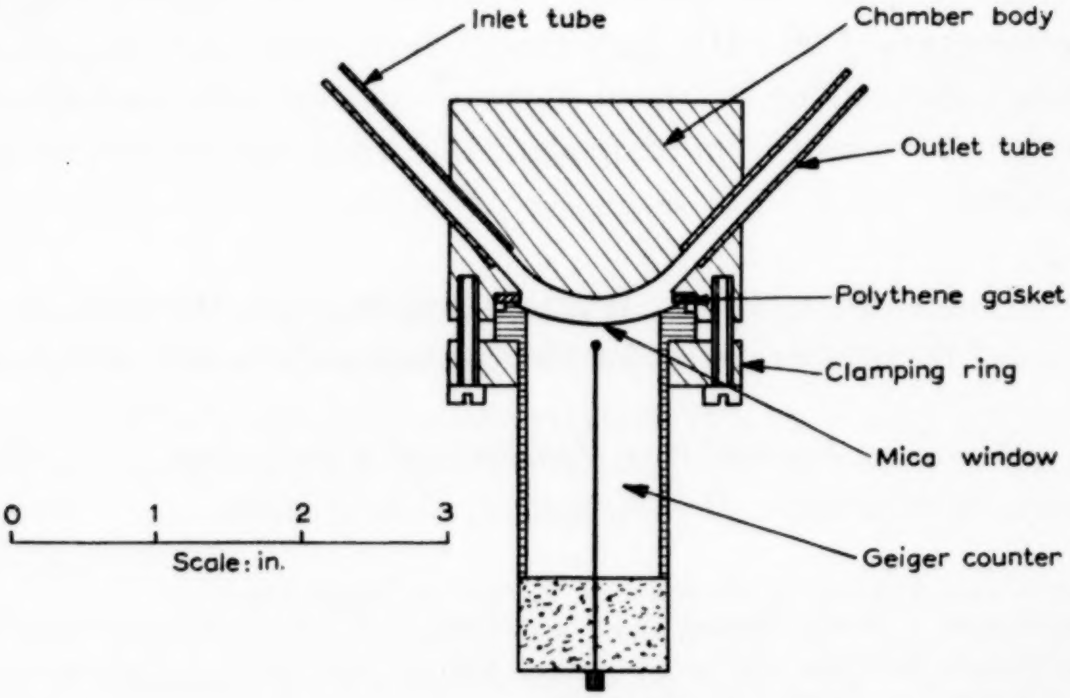

Fig. 1. Scale diagram of the assembly for monitoring radioactive effluents.

Performance. A large variety and volume of organic solvents have been passed over the window of the counter without perceptibly affecting its performance. After

6 months of operation, in which the counter was often in use continuously for periods of up to 24 h, the shape of the plateau and background were unchanged. The background was 13–14 c.p.m. when the assembly was mounted inside a 2 in. lead castle (standard A.E.R.E. type 1364A) as shown in Fig. 2. When charged with an ether solution of ^{14}C-labelled alcohol, the count rate was reduced to 58%, 26%, and 0.3% of the original after passing 1.2 ml, 2.5 ml, and 5.0 ml, respectively, of inactive solvent through the cell; after passing 7.5 ml, the count rate was reduced to background. Fig. 3 shows the trace obtained by passing 50 ml of each of the following solutions successively through the detector, each over a period of 15 min, using an integrating time constant of 0.1 sec:

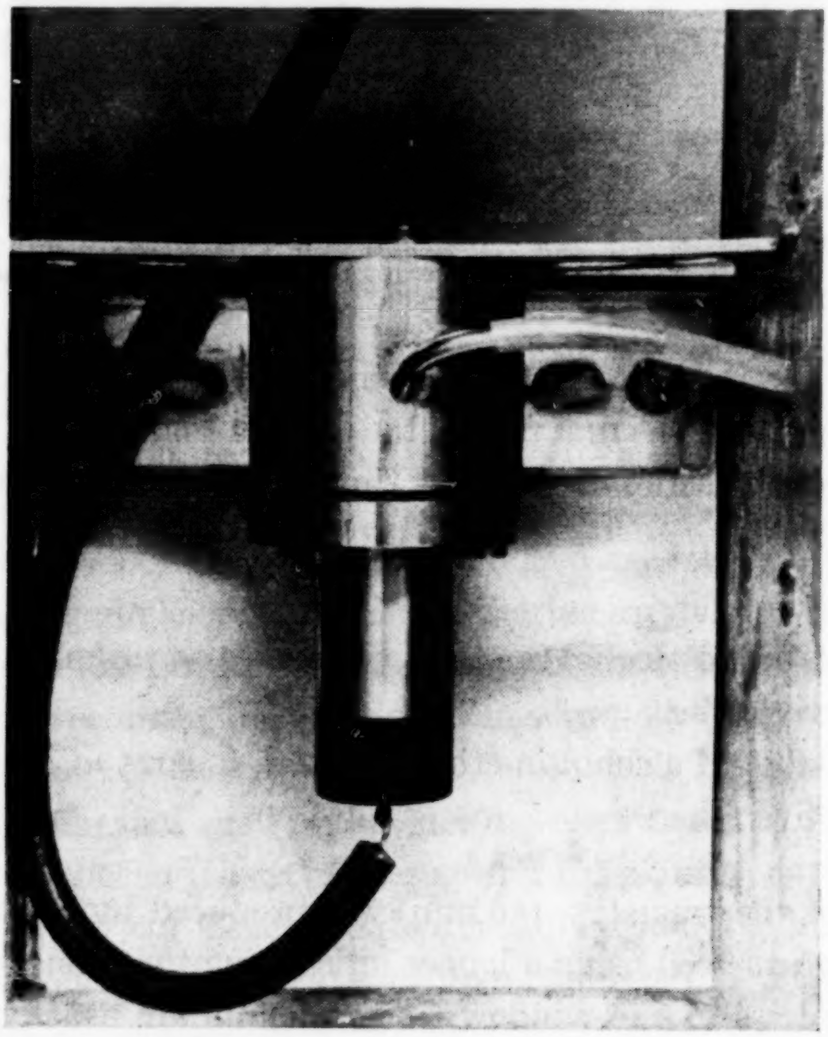

Fig. 2. Photograph showing the assembly mounted inside a 2 in. lead castle; the inlet tube passes through a hole in the back of the castle, the outlet through a hole in the side.

(a) Inactive ether,
(b) ^{14}C-labelled alcohol in ether solution (0.5 μC/ml),
(c) Inactive ether,
(d) ^{14}C-labelled alcohol in ether solution (0.25 μC/ml),
(e) Inactive ether.

In order to obtain a smooth curve and stable base line (background) when working at the lower limit of the detector it is necessary to use a longer integrating

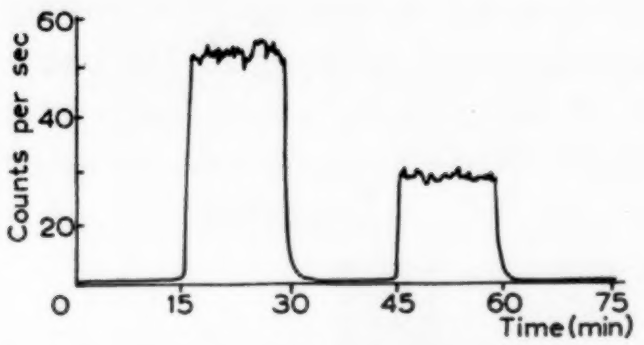

Fig. 3. Peaks obtained by passing solutions of ^{14}C-labelled alcohol at specific activities of 0.5 and 0.25 μC/ml, through the cell.

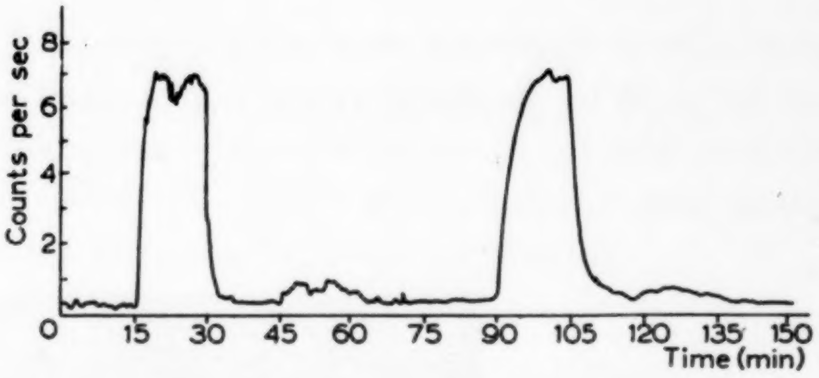

Fig. 4. The effect of different integrating time constants on the shape of the trace when solutions of specific activity 0.05 and 0.0025 μC/ml are passed through the cell; for 0–75 and 75–150 min the integrating time constants are 40 and 160 sec respectively.

time constant for the ratemeter, and this limits its sensitivity to small changes of activity of short duration. The first part of the trace in Fig. 4. (0–75 min) was produced by passing the following series of solutions through the cell using an integrating time constant of 40 sec:

(a) 50 ml inactive ether,
(b) 50 ml ^{14}C-labelled alcohol in ether solution (0.05 μC/ml),
(c) 50 ml inactive ether,
(d) 50 ml ^{14}C-labelled alcohol in ether solution, (0.0025 μC/ml),
(e) 50 ml inactive ether.

The second part of the trace (75–150 min) was produced by passing the same series of solutions through the cell using a longer integrating time constant (160 sec).

The mica windows of end-window Geiger counts are usually sealed in position with "Araldite", an expoxy resin that is very resistant to chemical attack. This type of assembly could be used for detecting β emitting isotopes other than ^{14}C, in organic or aqueous solutions providing they do not attack the metal or become adsorbed onto the mica window or surroundings.

Although intended primarily for measuring changes in activity, the cell can also be used for measuring the specific activity of solutions as the geometry is constant if the cell is always filled to capacity. It can easily be cleaned by flushing with solvent.

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Zur Trägerelektrophorese mit höheren Spannungen

In den letzten Jahren hat man in vielen Laboratorien zunehmend von der Möglichkeit Gebrauch gemacht, elektrisch geladene Stoffe (Ionen und Makromoleküle) durch Trägerelektrophorese unter Anwendung von Hochspannung bei gleichzeitiger Kühlung des Trägers zu trennen. Der Träger (Filterpapier, Stärke und viele andere faserige, pulverige oder gelartige Stoffe) wurde hierbei entweder durch ein indifferentes organisches Lösungsmittel, in welchem er frei hing (Filterpapier[1-3]), oder durch direkten Kontakt mit einer kalten Unterlage gekühlt. Als Unterlage benutzte man dabei meistens eine gekühlte Glas- oder Kunststoffplatte[4-8]. Von einer praktischen Verwendung von gekühlten Metallplatten, die wegen ihres hervorragenden Wärmeleitvermögens weitaus günstiger gewesen wären, hat man jedoch früher abgesehen, weil das Problem, die Metallplatte dauernd gegen Hochspannung zu isolieren, nicht befriedigend zu lösen war.

Da die Industrie jedoch heute durchaus in der Lage ist, Folien mit den erforderlichen mechanischen und elektrischen Eigenschaften zu liefern, erschien es lohnend, die als gekühlte Unterlage benutzten Glas- oder Kunststoffplatten durch eine Kupfer- oder Messingplatte zu ersetzen, die mit einer Kunststoffolie (z.B. 0.10 mm Hostaphan) überzogen worden war.

Ein Pherographiekasten aus Vinidur, der in seinen äusseren Abmessungen einem bekannten handelsüblichen Gerät (Pherograph-Frankfurt) entspricht, wurde so eingerichtet, dass hierin ein sicheres Arbeiten auf rationell gekühlter Metallplatte, die durch eine geeignete Folie isoliert wurde, möglich ist. In Fig. 1 ist die Gesamtansicht des Kastens wiedergegeben. Die Folie reicht bis zur halben Höhe der Seitenflächen des Kastens. Auf beiden Seiten wird sie durch zwei Klammern eingespannt.

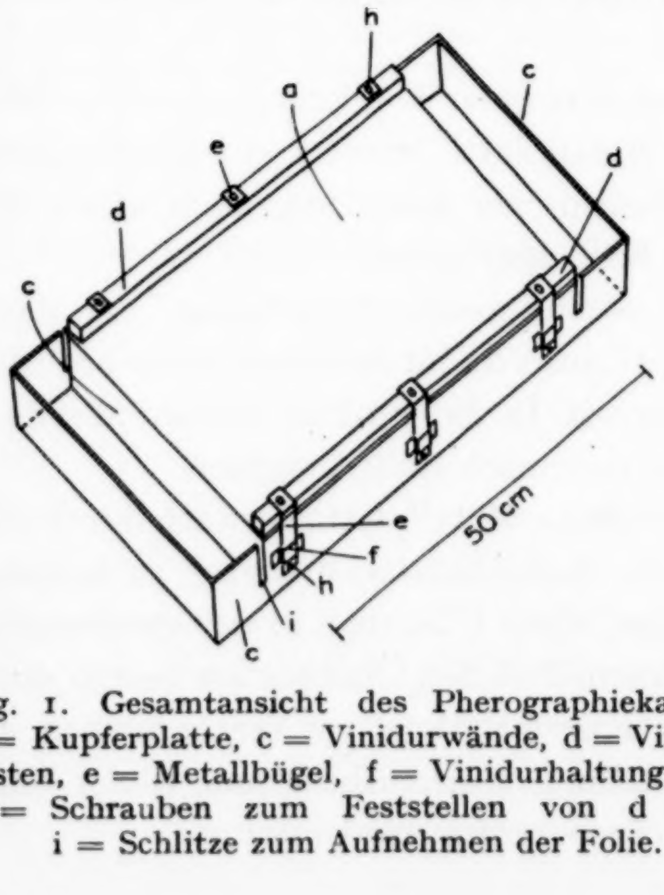

Fig. 1. Gesamtansicht des Pherographiekastens. a = Kupferplatte, c = Vinidurwände, d = Vinidurleisten, e = Metallbügel, f = Vinidurhaltung für e, h = Schrauben zum Feststellen von d usw., i = Schlitze zum Aufnehmen der Folie.

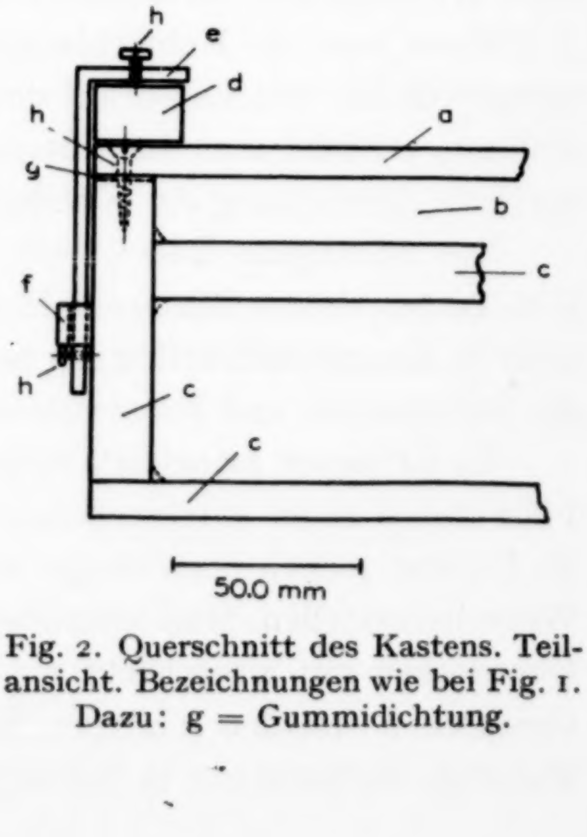

Fig. 2. Querschnitt des Kastens. Teilansicht. Bezeichnungen wie bei Fig. 1. Dazu: g = Gummidichtung.

Aus Fig. 2 (Querschnitt) erkennt man die Wirkungsweise dieser Klammern. Es ist zweckmässig, den Raum für die Kühlflüssigkeit möglichst klein zu halten, damit ein rascher Wechsel der Kühlflüssigkeit gewährleistet ist. Daher ist der Raum, den die Kühlflüssigkeit von der Stirnseite zu Stirnseite frei durchströmt, nur etwa 2 cm hoch (siehe Fig. 3). Es ist zweckmässig, die polierte Kupferplatte vor dem Aufbringen der Folie mit einem guten Ramsay-Fett leicht einzufetten; Luftblasen und ein stellenweiser Überschuss des Fettes werden entfernt, indem man mit einer planen Leiste (runde Kanten) vorsichtig, aber fest von einer Seite des Kastens zur anderen langsam über die Folie fährt.

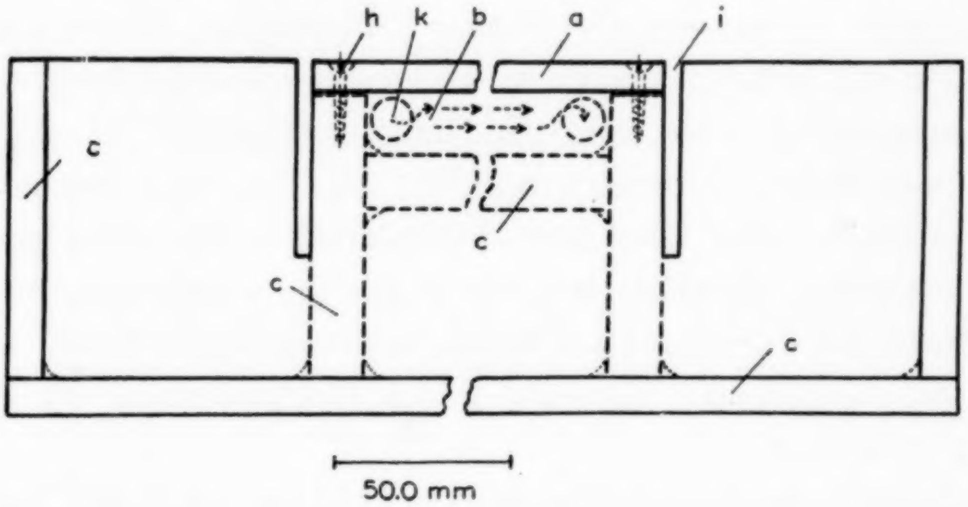

Fig. 3. Längsschnitt des Kastens. Bezeichnungen wie bei Fig. 1. Dazu: k = Rohr mit mehreren Öffnungen zur Zu- und Ableitung der Kühlflüssigkeit.

Die beschriebene Anordnung ist zum Betrieb mit "Kühlsole" (Gemische von Glycol bzw. Methanol mit Wasser) geeignet. Ist die abzuführende Joule'sche Wärme jedoch kleiner als 150 W (z.B. 100 mA bei 1500 V), kann man die Apparatur schon mit kaltem Leitungswasser (+ 12°) kühlen, so dass man bei diesen Ansprüchen auf das teure Kühlaggregat verzichten kann.

Wenn man die Rohrschlange des Verdampfers einer Kühlanlage in engen Windungen direkt von unten auf eine stärkere Metallplatte (etwa 1 cm Kupferplatte) auflötet, erreicht man bei entsprechender thermischer Isolierung nach unten eine optimale Ausnutzung der Kühlleistung eines Kälteaggregates.

Das wichtigste Bauelement der zuerst beschriebenen Anordnung: der flache Kühlkasten, dessen Seitenwände und Unterseite aus Vinidur bestehen, kann natürlich auch in Ganzmetallausführung hergestellt werden. In diesem Fall müssen allerdings die Seitenwände und Unterseite entsprechend thermisch isoliert werden.

Es ist ferner prinzipiell möglich, die erwähnte Metallplatte anstatt durch eine Folie durch einen entsprechenden Nylon- oder Kunstharzlacküberzug[9] zu isolieren. Es bereitet jedoch noch einige Schwierigkeiten, diese Überzüge in der gewünschten Weise herzustellen. Man wird aber auch in diesem Fall den Überzug am besten durch Überdecken mit einer leicht austauschbaren Kunststofffolie vor mechanischen und chemischen Schäden schützen, da eine Herstellung und Ausbesserung dieser Lacküberzüge vorläufig nur in Spezialbetrieben vorgenommen werden kann.

Die Vorteile der geschilderten Anordnungen bestehen also vor allem in der optimalen Ausnutzung der zur Verfügung stehenden Kälteleistung. Eine Bruchgefahr besteht nicht. Sollte die Folie durch mechanische oder chemische Einwirkungen beschädigt worden sein, so kann sie innerhalb von 15 min durch eine andere ersetzt werden. In der Regel kann aber monatelang mit derselben Folie gearbeitet werden. Gleich gute Erfahrungen mit den geschilderten Anordnungen wurden bereits auch in anderen Laboratorien* gemacht.

Da bei der hier angegebenen Wahl Metallplatte als gekühlte Unterlage auch grössere Mengen an Joule'scher Wärme abgeführt werden können, kann auch in elektrolytreicheren Pufferlösungen gearbeitet werden (Ionenstärke bis 0.5). Auf diese Weise ist es möglich, in dem gleichen Träger wesentlich grössere Substanzmengen aufzutrennen als es bei anderen Verfahren der Fall ist, da bei Verwendung einer grösseren Elektrolytkonzentration auch die Konzentration der zu trennenden Ionen erhöht werden kann, ohne dass das elektrische Feld an diesen Stellen wesentlich verkleinert wird. So lässt sich z.B. die bei pH 1.9 in einem Ameisensäure–Eisessig-Puffer (5% Ameisensäure, 15% Eisessig) auftrennbare Menge an Aminosäuren verdreifachen, wenn die Ionenstärke dieser Pufferlösung (und damit ihre Leitfähigkeit) durch Zusatz von Triäthanolammonium-trichlor-acetat etwa verdreifacht wird.

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Farbnachweis von Peroxydase auf Pherogrammstreifen[*]

Der färberische Nachweis von Katalase auf dem Papier mit 0.5%iger Wasserstoffsuperoxyd-Lösung als Substrat und anschliessendem Besprühen mit $FeCl_3$–$K_3Fe(CN)_6$-Lösung[1,2] erwies sich nach unseren Erfahrungen als nicht immer zuverlässig. Wir arbeiteten daher die kolorimetrische Reaktion nach KAUL[3] auf Verwendbarkeit auf dem Papier aus. Ein Fermentextrakt (0.1 ml), z.B. aus Weizenkeimlingen, lässt sich bei pH 4.6 und 110 V innerhalb von 14–16 h bei 6° papierelektrophoretisch gut trennen. Der Extrakt wird strichförmig am Start aufgetragen. Als Puffer zur Tränkung des Papierträgers dient ein Acetat-Puffer (49 Teile einer 0.2 *M* Natriumacetat-Lösung und 51 Teile 0.2 *M* Essigsäure). Der ohne Erhitzung luftgetrocknete Streifen wird nach der Trennung mit folgender Lösung (A) besprüht:

Lösung A: 2 ml Natriumacetat 0.2 *M*
0.25 ml Lösung B
0.1 ml $MnSO_4$ 0.005 *M*.

Es kann zweckmässig sein diese Lösung mit Natriumacetat auf pH 4.6 einzustellen.

0.1 ml H_2O_2-Lösung, hergestellt durch Verdünnen von 0.1 ml 30%igen Wasserstoffperoxyds (Perhydrol Merck) in 25 ml quarzdestilliertem Wasser.

Lösung B, hergestellt unter Erhitzen auf 30–40°: 50 mg Benzidin
135 mg Guajakol
25 ml 10%ige Essigsäure.

Die Lösung A ist stets frisch vor der Verwendung zusammenzustellen, die verdünnte Wasserstoffperoxyd-Lösung wird unmittelbar vor dem Sprühen zugefügt.

Lösung B ist *ca.* 4 Wochen lang im Kühlschrank bei 1–3° haltbar. Nach einigen Tagen auftretender brauner Niederschlag kann ohne Beeinträchtigung der Wirksamkeit abzentrifugiert werden.

Der besprühte Pherogrammstreifen weist nach *ca.* 5 min bei Zimmertemperatur unter Vermeidung direkter Beleuchtung (diffuses Licht) die Peroxydase enthaltenden Fraktionen als rot gefärbte Streifen auf. Die Intensität der Farbreaktion ist stark pH-abhängig. Das Optimum liegt bei pH 4.6. Die Farbreaktion ist mehr als 24 h haltbar.

Eine quantitative Erfassung der Reaktion ist möglich: der Farbstoff kann nach 5 min aus dem Papierabschnitt, der die Katalase-Fraktion enthält, mit einem Alkohol–Chloroform-Gemisch (1:9) durch einmaliges Ausschütteln (*ca.* 30 sec) rückgelöst werden und ist dann haltbar. Die quantitative Bestimmung erfolgt unter Berücksichtigung einer Blindprobe photometrisch bei 510 mμ an Hand einer empirischen Eichkurve.

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[*] Herrn Prof. J. Schwemmle (Erlangen) zum 65. Geburtstag gewidmet.

BOOK REVIEWS

Gas Chromatography 1958 (Proceedings of the Second Symposium organised by the Gas Chromatography Discussion Group under the auspices of the Hydrocarbon Research Group of the Institute of Petroleum, and the Koninklijke Nederlandse Chemische Vereniging, held at the Royal Tropical Institute, Amsterdam, 19th–23rd May, 1958), edited by D. H. DESTY. Published by Butterworths Scientific Publications, London, 1958, pp. xiii + 383, price 70 s; American edition by Academic Press Inc., New York, price $ 12.00.

The international aspect of the Symposium is demonstrated by the 28 specialist papers contributed from 9 countries, together with 3 short introductory papers by internationally known workers in the field—the short paper by Dr. A. J. P. MARTIN on Recent Trends and New Developments being a particularly good review.

The papers are divided into three Sections: I. Theory of Gas Chromatography; II. Techniques and Apparatus; III. Applications. The discussions on the papers are given in full and are a valuable record of the informal exchange of ideas which takes place at such a Symposium. For these discussions an author index and a brief subject index are given, the latter being a useful and unusual feature of a book of this kind. However, the reviewer thinks that a subject index for the original papers would have added very considerably to the value of the book for reference purposes. Recommendations on nomenclature are briefly mentioned but show little alteration to those put forward at the London Symposium in 1956.

The papers in Section I deal with the theory of the attainment of high efficiency in both packed and capillary gas–liquid columns, the theory of chromatography of highly radioactive gases, the correlation of sorption isotherms with the chromatographic behaviour of vapours, the achievement of symmetrical elution curves in adsorption chromatography, a hydrodynamic model of a sorption column, and the determination of activity coefficients at infinite dilution. While the theory of the conventional packed gas–liquid column is only slowly being consolidated and interpreted, a detailed theory has been put forward for the most recent development, the coated capillary column. This new weapon in the already impressive armoury of the gas chromatographer offers great improvements for the future in terms of high efficiency and rapid separations. In the meantime the vast store of practical experience that is being accumulated with "conventional" columns is well demonstrated by the contents of Sections II and III.

Papers in Section II give details of various detectors based on flame ionisation, emissivity, the hydrogen flame, and thermal conductivity. Useful practical details are given of the construction of highly efficient packed columns, of the use of multiple columns and programmed heatings, of base-line control, of operating data on two stationary phase supports, and of quantitative gas-analysis with thermal conductivity detection.

In Section III some stimulating and novel applications of gas chromatography are described and these are sure to have a great impact on a number of fields of research. Examples are the analysis of traces of hydrogen in water (applicable to the control of corrosion in high pressure boilers), volatile halogen and inter-halogen compounds, essential oils, derivatives of amino-acids; the separation of phenols using sugars as stationary phases; and the increasing refinement of automatic preparative-scale apparatus. The final paper discusses the presentation of retention volume data.

The printing and illustrations are of the very high standard which one has come to associate with Butterworths Scientific Publications and there are remarkably few errors. The editor, the publisher, and the printers must be congratulated on their successful co-operation and prompt production of these Proceedings. The volume is essential for reference libraries and is also strongly recommended to individual workers for its wealth of practical information for day-to-day use.

C. J. HARDY (Harwell)

Eletroforese em papel e métodos relacionados, by LUIZ PAULO RIBEIRO, EMILIO MITIDIERI AND OTTILIA R. AFFONSO with an introduction by GILBERT VILLELA. Serviço Gràfico do I.B.G.E., Rio de Janeiro, 1958, 553 pp., 129 figs., 42 tables.

This book is to be welcomed not only because it is the first treatise on this subject to be published in Portuguese, but also because considerable effort has been expended in making it a comprehensive review of all the principles and techniques of paper electrophoresis. It contains more than 2500 literature references.

The book consists of three main parts: the first part reviews the theory of paper electrophoresis (pp. 1–27), and the methods and apparatus employed (pp. 28–47). The second part (Chapters IV–XV, pp. 48–247) is devoted to the electrophoresis of proteins and its clinical applications, while Chapters XVI, XVII, XVIII and XX (pp. 248–350 and pp. 365–385) of the third part deal with electrophoresis of non-macromolecular substances, such as hormones, vitamins, nucleic acids, amino acids, alkaloids and amines, and inorganic ions. Immunochemistry and immuno-electrophoresis are discussed in Chapter XIX, where particular emphasis is laid on these new methods.

All aspects of paper electrophoresis are excellently reviewed, and critically examined by a group of workers who have spent several years studying biochemical problems by means of these techniques.

The book is provided with an author and a subject index, which is very useful for reference purposes.

The printing is very good and a great number of photographs clearly illustrate the text.

G. B. MARINI-BETTÒLO (Rome)

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REVIEW

THE CHROMATOGRAPHY OF THE FLAVONOID PIGMENTS

J. B. HARBORNE

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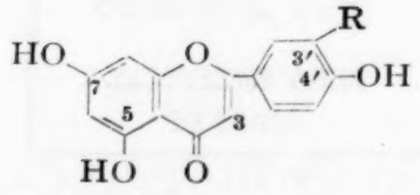
INTRODUCTION

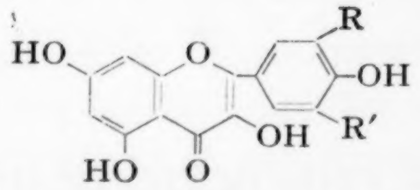
Paper partition chromatography was first applied to the separation of flavonoid pigments by BATE-SMITH[1,2] in 1948. Since then, it has been used increasingly in the study of these compounds, which are ideally suited to this particular technique. The flavonoids have just the right range of solubility characteristics for ease of separation and most of them possess characteristic colours on paper in visible or ultraviolet light. Paper chromatography has thus provided the first satisfactory means of surveying plant material for the presence of these water-soluble pigments[3,4]. It has been particularly valuable for separating the components of mixtures of flavonoids, which occur in some plants (*e.g.*[5,6]). Large scale separations have been carried out by means of adsorption chromatography on cellulose or magnesol columns[7,8]. But it is in the characterisation and elucidation of structure of unknown flavonoids on a micro-scale that chromatography has been so successful[5,6,9,10]. The identification of flavonoids by means of chromatography is therefore the major topic of this review. A large number of R_F values are given and some new ones determined in this laboratory are included.

The term flavonoid is used here to describe that group of water-soluble phenolic pigments sometimes known as the anthoxanthins. These are the widely occurring flavones and flavonols and the less frequently distributed flavanones, isoflavones, chalcones and aurones. Flavans (monomeric leucoanthocyanins and catechins) and other related substances may also be considered to fall into this category. The chromatography of the anthocyanins, which are associated with and are structurally related to the anthoxanthins, has already been discussed in this journal[11].

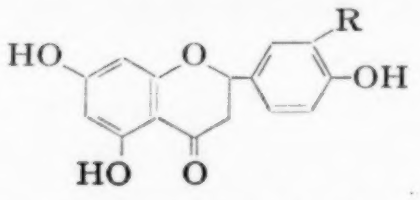
In order to be able to relate R_F value to structure (section 3), a brief consideration of the chemical structure of the flavonoids must be made. The formulae of some of the commonly occurring compounds are shown in Fig. 1. Examples are included of all the main classes of flavonoids, which generally differ from each other in the oxidation level of the central pyran ring. Within these classes, structural variation is mainly confined to differences in the number and position of hydroxyl, methyl and glycosyl substituents. There are also a considerable number of rare flavonoids, with additional substituents (*e.g.* methylene-dioxy groups, isoprene side chains, etc.). Their structures are shown in a recent review of the naturally occurring flavonoid pigments by GEISSMAN AND HINREINER[12].



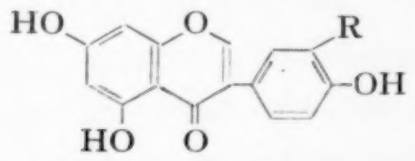
Flavones: apigenin (R = H), luteolin (R = OH)



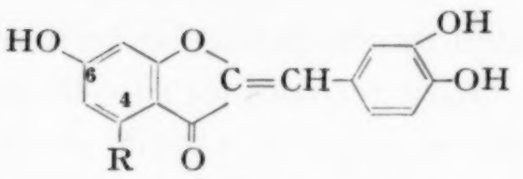
Flavonols: kampferol (R = R′ = H), quercetin (R = OH, R′ = H), myricetin (R = R′ = OH)



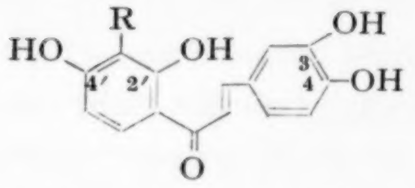
Flavanones: naringenin (R = H), eriodictyol (R = OH)



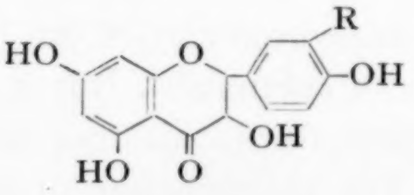
Isoflavones: genistein (R = H), orobol (R = OH)



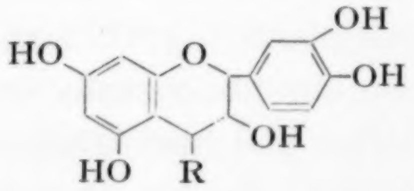
Aurones: sulphuretin (R = H), aureusidin (R = OH)



Chalcones: butein (R = H), okanin (R = OH)



Flavanonols: dihydrokampferol (R = H), dihydroquercetin (R = OH)



Flavans: catechin (R = H), leucocyanidin (R = OH)

Fig. 1. Commonly occurring flavonoids.

I. CHROMATOGRAPHIC TECHNIQUES

Bate-Smith[2, 13] measured the R_F values of thirty-six flavones in two solvent systems. Wender and his associates[14, 15] chromatographed a larger selection of compounds in eleven solvent systems and detected the flavone spots on paper with seven chromogenic sprays. Numerous other workers (*e.g.* Paris[16], Geissman[17]) have described procedures for the chromatography of the flavones. Brief summaries have appeared in manuals of chromatography[18, 19].

Extraction

Flavones are commonly extracted from fresh plant material with hot 70 % ethanol, 95 % ethanol, methanol or water. By plunging the plant material into boiling solvent, the enzymic hydrolysis of glycosides can be kept to a minimum[20]. The extracts are filtered, concentrated to a small volume *in vacuo*, re-filtered and applied by means of a capillary or a micropipette to the chromatography paper. Leaf extracts are first washed with petroleum ether to remove chlorophyll because this compound may cause flavones to streak on chromatograms. Samples of flavones are dissolved in ethanol or 70 % ethanol, if necessary by heating, before being applied to paper.

Filter paper

Whatman No. 1 paper (chromatography grade) is widely used for the chromatography of flavones. Thicker papers (Whatman No. 3 or 3 MM) are suitable for small scale separations. Papers buffered either with borate or phosphate[21] have been used by NORDSTRÖM AND SWAIN[5] for the separation of partially methylated flavones. SEIKEL[22] has pointed out that the R_F value varies with the pH of the buffer. This pH should always be given.

Solvent systems

A very large number of solvent systems have been used for the separation of flavonoids on paper.

(1) *Aqueous solvents.* Water is itself an excellent solvent for catechins[23] and flavonol glycosides[24]. It is useful for separating many glycosides from their aglycones, which are usually immobile. Furthermore, those aglycones which travel in water (*e.g.* flavanones) can be separated from those that do not (*e.g.* flavones, flavonols). Some compounds tend to streak with distilled water but this can be minimized by adding acetic acid (2 % v/v)[24] or inorganic salt (3 % w/v sodium chloride)[22]. Stronger mixtures of acetic acid with water are most valuable for separating flavone and flavonol aglycones[4]; the Forestal solvent system (acetic acid–conc. HCl–water, 30:3:10, by vol.), 30 % acetic acid and 60 % acetic acid are examples.

(2) *Alcoholic solvents.* The upper phase of the mixture of *n*-butanol–acetic acid–water (4:1:5, by vol.) (designated as BAW) is most valuable for separating all types of flavonoids, both glycosides and aglycones. Single phase mixtures of these solvents, *i.e.* the proportions 6:1:2[5] or *n*-butanol–27 % acetic acid (equal volumes)[6] have also been used. Alcohol–acetic acid mixtures are sensitive to small temperature changes and alter in composition with age. R_F values determined in BAW often vary more than those measured in other kinds of solvents (*e.g.* aqueous systems).

Other solvent systems containing alcohols have been used to a lesser extent. Examples are *n*-butanol–water (1:1, v/v; upper phase)[14], water-saturated *sec.*-butanol[25] and *n*-propanol–acetic acid–water (1:1:1, by vol.)[26].

(3) *Phenolic solvents.* Phenol or *m*-cresol saturated with water and *m*-cresol–acetic acid–water (50:2:48, by vol.)[2] give a greater spread of R_F values and alter the relative positions of flavones on chromatograms, as compared with BAW. A certain amount of streaking takes place on papers developed with phenolic solvents. Owing to their intense absorption in the ultraviolet, phenols are not suitable solvents for use in purifying flavones on paper for spectral measurements.

(4) *Hydrocarbon solvents.* Benzene–ligroin, b.p. 85–105°–methanol–water (50:50:1:50, by vol.) and related systems devised by LINDSTEDT AND MISIORNY[27] are reported to give excellent separation of flavonoids containing one to three hydroxyl groups, *e.g.* those present in the heartwood of pines. On the other hand mixtures of

chloroform–isopropyl alcohol–water, chloroform saturated with water and ethyl acetate saturated with water cannot be recommended for general use, as considerable streaking occurs in these systems[15].

Methods of development

The usual technique of one-dimensional chromatography by descent is commonly used. Suitable solvent pairs for two-dimensional chromatography are BAW and 2% acetic acid[10], *n*-butanol–27% acetic acid (1:1, v/v) and *m*-cresol–acetic acid–water (50:2:48, by vol.)[6,17], and ethyl acetate–acetic acid–water (50:2:50, by vol.) and BAW[28]. Circular paper chromatography is another technique which can be used with flavones[29]. Paper electrophoresis will separate flavones on Whatman No. 4 paper in *n*-butanol–ethanol–borate buffer (1:1:1, by vol.)[30].

Visualisation

Some flavonoids (*i.e.* aurones and chalcones) can be seen on paper chromatograms as yellow spots in daylight. In ultraviolet light the majority appear as intensely coloured spots, which may be fluorescent. Flavonoids containing free phenolic groups undergo a characteristic and reversible colour change when the papers are fumed with ammonia vapour in ultraviolet light. Some compounds (*e.g.* flavans, isoflavones) can only be detected by means of chromogenic sprays. Among the most useful of these are 5% ethanolic aluminium chloride[31], 5% aqueous sodium carbonate, 1% sodium borohydride in isopropanol[32] and a fresh mixture of equal volumes of 1% aqueous ferric chloride and 1% aqueous potassium ferricyanide[33]. After spraying with aluminium chloride or carbonate, the chromatograms should be dried and examined in ultraviolet light, as these reagents impart a fluorescence to many flavonoids. The response of a typical member of each class of flavonoid compound to these different methods of detection is shown in Table I.

Flavonoids in the same structural class often give characteristic colour reactions. For example, only flavanones form red or magenta colours on reduction with sodium borohydride and subsequent exposure to acid vapour[32]. On paper flavonols and flavones give a particularly distinctive range of colours in the ultraviolet, with and without ammonia vapour[4]. Flavones with a free 3-hydroxyl group (*i.e.* flavonol aglycones) are characteristically bright yellow whereas those without one (*i.e.* flavonol 3-glycosides and ordinary flavones) are dull brown in ultraviolet light. Flavonols without a 5-hydroxyl group (*e.g.* fisetin, robinetin) or with a methylated 5-hydroxyl group (*e.g.* azaleatin) have a greenish-yellow or yellow fluorescence. Flavonols with both the 3- and 5-hydroxyl groups substituted (*e.g.* kampferol tetramethyl ether) fluoresce blue[34,35].

In practice, it is not always possible to classify flavonoids on the basis of their colour reactions alone. This is because some are exceptional in their behaviour. Some of the highly hydroxylated flavonols (*e.g.* quercetagetin, 3,5,6,7,3',4'-hexahydroxyflavone) are dull brown rather than bright yellow in ultraviolet light[4]. Other flavonols, *i.e.* those with hydroxyl groups in the 3', 4' and 5' positions such as myricetin, give a

TABLE I

THE COLOUR REACTIONS OF FLAVONOIDS ON PAPER

Reagent	*Light source*	*Class of flavonoid*					
		Flavone	*Flavonol*	*Isoflavone*	*Flavanone*	*Aurone*	*Chalcone*
None	visible	pale yellow	pale yellow	colourless	colourless	bright yellow	yellow
None	ultraviolet	dull brown	bright yellow	faint purple	colourless	bright yellow	dull brown
NH_3 vapour	ultraviolet	bright green	bright yellow	faint purple	colourless or pale yellow	bright red	dull red
$AlCl_3$	visible	pale yellow	yellow	colourless	colourless	pale yellow	yellow
$AlCl_3$	ultraviolet	fluorescent green	fluorescent yellow	fluorescent yellow	fluorescent green	fluorescent green	fluorescent orange
Na_2CO_3	visible	bright yellow	yellow	pale green	green	orange	red
$NaBH_4$	visible	colourless	colourless	colourless	magenta	colourless	colourless
$FeCl_3$–$K_3Fe(CN)_6$	visible	blue	blue	blue	blue	blue	blue

blue and not a yellow colour (Table I) after spraying with sodium carbonate solution. Also flavones with a methyl substituent on the 4′-hydroxyl group do not give the usual colour change in ultraviolet light on fuming with ammonia. Again, 7,8-dihydroxyflavanones are different from the others. They fluoresce bright blue on paper when fumed with ammonia in ultraviolet light[9]. Finally, as there is no specific test for isoflavones, they cannot be readily distinguished from flavans.

Elution from paper

In studying flavonoids by paper chromatography, it is necessary to have an efficient method of removing the pigments from paper for purification and further study. A useful general solvent is ethanol containing 30% water. Elution of short strips of paper containing the pigment by capillary flow from a central reservoir is best carried out in a closed vessel of the type described by GAGE AND WENDER[31]. The rate of elution is followed by removing a strip, drying it and examining it in ultraviolet light. Elution usually takes from 4 to 16 hours. With strongly adsorbed pigments the addition of acetic acid (10% by vol.) to the eluting solvent is recommended.

It is not advisable to keep flavonoids stored indefinitely on paper strips as chemical changes may take place. For example, a chalcone glycoside of *Coreopsis maritima* was converted to the corresponding aurone glycoside, when kept on Whatman No. 3 paper for several weeks[9]. The hydrolysis of glycosides on paper has been noted frequently in this laboratory.

Sources of standard markers

As already mentioned, standard markers should be run on all chromatograms. An unknown can be identified provisionally if its mixture with a sample compound does not separate when chromatographed in several solvent systems. For these purposes, samples of a reasonable number of authentic flavonoids are required. Fortunately, many of the more common flavonoids such as rutin, naringin, hesperidin and quercitrin are available commercially. Other flavonoids can easily be obtained from them by simple chemical procedures (*e.g.* acid hydrolysis, methylation, oxidation etc.). For example, quercetin can be obtained from the commercially available glycosides rutin and quercitrin by acid hydrolysis.

A number of flavonoids can be obtained from common vegetable materials. For example, "instant tea" is a source of kampferol, quercetin and myricetin[36]. Parsley seed may be extracted to obtain apiin and a hydrolysed extract contains apigenin and luteolin[37]. Roots of *Iris tectorum* and *Iris florentina*, the latter being sold as orris root in powder form, provide tectiridin and iridin respectively[4]. Medicines of vegetable origin may also be useful. Thus hyssop has been used as a source of diosmin[4].

Column chromatography

The large scale separation has been carried out on columns of silica-gel[38], magnesol (a magnesium trisilicate)[8], cellulose[7], celite[39], ion-exchange[40] or polyamide resins[41, 42]. Of these absorbents, magnesol has been most used. In a typical procedure[8], a column

6 cm in diameter and 16 cm deep is prepared from a slurry of magnesol in acetone. After washing the column with acetone, the plant extract in the same solvent is applied to it and development proceeds with water-saturated ethyl acetate. Flavonoid bands are visible on the column, when viewed in ultraviolet light. The pigments are collected in separate fractions as elution proceeds. Very strongly adsorbed pigments may have be to extruded from the column.

2. THE R_F VALUES OF FLAVONOIDS

Several hundred natural flavonoid compounds are known[12,43] and additions are regularly being made to this total. A tabulation of the R_F values of all of them cannot be made as the data available in the literature are by no means comprehensive or strictly comparable. Most of the R_F values in Tables II–VIII have been determined in this laboratory in order that they may be compared directly with each other. The R_F values of some of the compounds are given here for the first time.

On the basis of their colour reactions on paper chromatograms, the flavonoids fall into three groups. There are the flavones and flavonols which appear in ultraviolet light as intense brown to yellow spots, which may be fluorescent. Then there are the flavanones, isoflavones and related compounds which are colourless or faint purple

TABLE II

R_F VALUES OF FLAVONE AND FLAVONOL AGLYCONES

Name	*Flavone substituents*	*R_F values in*			*Colours in ultraviolet*
		BAW	*Forestal*	*Phenol*	
Flavones					
Acacetin	5,7-diOH,4′-OMe	0.91	0.91	0.88	dark brown
Apigenin	5,7,4′-triOH	0.89	0.83	0.88	dark brown***
Diosmetin	5,7,3′-triOH,4′-OMe	0.85	0.80	0.86	dark brown
Luteolin	5,7,3′,4′-tetraOH	0.78	0.66	0.66	dark brown***
Tricin	5,7,4′-triOH,3′,5′-diOMe	0.73	0.72	0.87	dark brown***
Saponaretin	5,7,4′-triOH,8-subst.*	0.56	0.86	0.80	dark brown***
Vitexin	5,7,4′-triOH,8-subst.**	0.43	0.82	0.63	dark brown***
Flavonols					
Kampferol	3,5,7,4′-tetraOH	0.83	0.55	0.58	yellow-green
Morin	3,5,7,2′,4′-pentaOH	0.79	0.73	0.34	yellow
Isorhamnetin	3,5,7,4′-tetraOH,3′-OMe	0.74	0.53	0.66	yellow
Fisetin	3,7,3′,4′-tetraOH	0.73	0.58	0.32	yellow-green FL.†
Rhamnetin	3,5,3′,4′-tetraOH,7-OMe	0.72	0.53	0.66	yellow
Quercetin	3,5,7,3′,4′-pentaOH	0.64	0.41	0.29	yellow
Azaleatin	3,7,3′,4′-tetraOH,5-OMe	0.48	0.49	0.50	yellow FL.
Myricetin	3,5,7,3′,4′,5′-hexaOH	0.43	0.28	0.13	golden yellow
Robinetin	3,7,3′,4′,5′-pentaOH	0.40	0.36	0.18	yellow-green FL.

* —$(CHOH)_5 \cdot CH_2OH$.

**

$—CHOH \cdot \underbrace{CH \cdot CHOH \cdot CHOH \cdot CH}_{O} \cdot CH_2OH.$

*** These flavones become bright green or yellow-green when fumed with ammonia; the other two are unchanged.

† FL. = fluorescent.

in ultraviolet light. Finally, there are the "anthochlor" pigments, the chalcones and aurones, which are visible in daylight as yellow spots, but which change to red when fumed with ammonia.

Flavones and flavonols

Table II shows the R_F values in three solvent systems of 16 flavone and flavonol aglycones. Five of them, myricetin, quercetin, kampferol, luteolin and apigenin are regularly found as glycosides in the flowers of higher plants. The three naturally occurring mono-methyl ethers of quercetin are included. Two of these, rhamnetin and isorhamnetin have very similar R_F values (see also BARBER[46]) but the third, azaleatin[47] has quite a different set of values from quercetin and the other two. The R_F values of vitexin and saponaretin are also given. These are two apigenin derivatives with hydroxylated side chains in the 8-position[48, 49]. They represent an unusual group of

TABLE III

R_F VALUES OF FLAVONOL GLYCOSIDES

Glycoside	*R_F values in*			
	BAW	*Water*	*15% acetic acid*	*Phenol*
*Kampferol**				
3-monoglucoside (astralagin)	0.70	0.13	0.43	0.74
3-rhamnoglucoside (nicotiflorin)	0.54	0.23	0.54	0.64
3-diglucoside**	0.43	0.27	0.54	0.55
3-triglucoside**	0.31	0.33	0.51	0.45
3-rhamnodiglucoside	0.41	0.34	0.61	0.54
3-rhamnogalactosido-7-rhamnoside (robinin)	0.40	0.54	0.75	0.73
3-rhamnoglucosido-7(?)-glucoside***	0.40	0.54	0.74	0.52
7-monoglucoside	0.54	0.02	0.17	0.62
7-monorhamnoside	0.75	0.02	0.18	0.76
3-xyloglucoside	0.55	0.29	0.65	0.68
Quercetin				
3-monoarabinoside (avicularin)	0.70	0.07	0.31	0.61
3-monoxyloside (reynoutrin)	0.65	0.06	0.32	—
3-monoglucoside (isoquercitrin)	0.58	0.08	0.37	0.54
3-monogalactoside (hyperin)	0.55	0.09	0.35	0.56
3-monorhamnoside (quercitrin)	0.72	0.19	0.49	0.58
3-rhamnoglucoside (rutin)	0.45	0.23	0.51	0.46
3-diglucoside**	0.37	0.19	0.45	0.36
3-triglucoside**	0.23	0.18	0.41	0.26
3-rhamnodiglucoside	0.36	0.26	0.54	0.35
3-rhamnoglucosido-7(?)-glucoside***	0.36	0.46	0.71	0.31
7-monoglucoside (quercimeritrin)	0.37	0.00	0.07	0.40
4'-monoglucoside (spiraeoside)	0.48	0.01	0.13	0.33
3,4'-diglucoside-3'-methyl ether (dactylin)	0.38	0.27	0.62	0.63
Myricetin				
3-monoglucoside** (cannabiscitrin)	0.47	0.05	0.25	0.32
3-monorhamnoside (myricitrin)	0.60	0.15	0.44	0.39

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* Colours under U.V. light with ammonia; kampferol glycosides: yellow-green; quercetin glycosides: yellow; myricetin glycosides: yellow-brown.

** Isolated from flowers of *Primula sinensis*.

*** Provisional structures for pigments isolated from flowers of *Solanum brachycarpum*.

flavones only found so far in seven species[49] and have different R_F values from the common flavones. For example, vitexin and saponaretin (R_F's 0.06 and 0.16 respectively) are the only flavones or flavonols except morin (R_F 0.03) which are mobile on paper with water as solvent.

The R_F values of flavones not included in the list are given in the papers of BATE-SMITH[2, 4], WENDER and co-workers[14, 15] and GEISSMAN[17]. Of these only quercetagetin, 3,5,6,7,3′,4′-hexahydroxyflavone (R_F 0.40 in BAW, 0.29 in Forestal) has been found in several species[4]. Flavone itself, which occurs on the surface of the leaves and stems of many *Primula* species, travels near the front in most solvent systems[17]. It appears as a light-absorbing spot against a fluorescent background in ultraviolet light of 253 mμ wavelength.

The R_F values of 25 representative glycosides of the commonly occurring flavonols are shown in Table III. Examples of glycosides with different sugars are included; they vary in number and position of substitution. In the case of quercetin, the R_F values of 5 different 3-monoglycosides, of 3-mono-, 3-di- and 3-triglucosides and 3,7- and 7-glycosides are shown. As with the anthocyanins[11], corresponding glycosides of kampferol, quercetin and myricetin can always be placed in the same order of R_F value. The approximate R_F value of an unknown glycoside in any one series may therefore be predicted with some confidence if the values for the other two members are known (see also[24]).

Table IV shows the R_F values of 11 flavone glycosides. As with the flavonols, the number and the position of attachment of the sugar residues affects the R_F value.

TABLE IV

R_F VALUES OF FLAVONE GLYCOSIDES

Glycoside	*R_F values in*			
	BAW	*Water*	*15% acetic acid*	*Phenol*
Apigenin[*]				
7-monoglucoside (cosmetin)	0.65	0.04	0.25	0.78
7-rhamnoglucoside (rhoifolin)	0.58	0.09	0.46	0.74
7-apiosylglucoside (apiin)	0.57	0.06	0.42	0.75
7-glucuronide	0.57	0.13	0.29	0.46
7-glucoside, 8-subst.[**] (saponarin)	0.42	0.31	0.64	0.65
4′-rhamnoside, 8-subst.[***] (of vitexin)	0.50	0.46	0.69	0.57
Luteolin				
7-monoglucoside	0.44	0.01	0.15	0.56
7-apiosylglucoside	0.42	0.03	0.23	0.50
7-diglucoside	0.40	0.05	0.29	0.54
5-monoglucoside (galuteolin)	0.82	0.00	0.07	0.65
Acacetin				
7-rhamnoglucoside (linarin)	0.61	0.14	0.60	0.55

[*] Colours in U.V. light with ammonia: apigenin glycosides: bright green; luteolin glucosides: yellow-green; acacetin glycoside: dull purple.

[**] See footnote[*], Table II.

[***] See footnote[**], Table II.

Thus luteolin 7-monoglucoside and 7-diglucoside are chromatographically distinct. Also the 5- and 7-monoglucosides of luteolin have different R_F values from each other (Table IV) and from those of the 4′-monoglucoside[50] (R_F 0.68 in BAW, 0.72 in phenol, 0.34 in 15% acetic acid). The R_F values in Table IV for luteolin 7-monoglucoside were measured on material isolated from several sources, including *Coreopsis maritima*[52], *Gesnera cardinalis*[54], *Solanum stoloniferum*[53] and *Dahlia variabilis*[54]. Another reported source of this compound, namely the leaves of *Digitalis purpurea*[51], contains two luteolin glycosides, both of which are quite different in their chromatographic behaviour from the 7-monoglucoside. One of the two compounds, for example, has an R_F of 0.30 in BAW, 0.12 in water and 0.12 in phenol saturated with water[54].

Table V gives the R_F values of hydroxypolymethoxy flavones derived from apigenin, luteolin, kampferol and quercetin. The use of these data in the determination of the position of sugar residues in flavones on a micro-scale[5] will be discussed later in section 6. For the separation of all the isomeric partial methyl ethers of luteolin and apigenin, NORDSTRÖM AND SWAIN[5] used buffered paper and a benzene–nitromethane–water mixture. R_F values of all the relevant methyl ethers of kampferol and quercetin

TABLE V

R_F VALUES OF METHYLATED FLAVONES AND FLAVONOLS

Methyl derivative	*R_F values in*			*Colours in ultraviolet*	
	C_6H_6–$MeNO_2$–H_2O (3:2:5)	*n-BuOH–H_2O*		*alone*	*with NH_3*
		borate paper	*phosphate paper*		
*Apigenin**					
4′,5-dimethyl	0.46	0.51	0.62	blue	yellow
4′,7-dimethyl	0.97	0.93	0.84	brown	brown
5,7-dimethyl	0.46	0.80	0.70	blue	green-blue
5,7,4′-trimethyl	0.91	0.87	0.79	blue	blue
*Luteolin**					
3′,4′,5-trimethyl	0.35	0.26	0.34	lilac	orange-yellow
3′,4′,7-trimethyl	0.95	0.92	0.77	brown	brown
3′,5,7-trimethyl	0.72	0.67	0.55	blue	yellow-green
4′,5,7-trimethyl	0.73	0.77	0.55	blue	red-purple
3′,4′,5,7-tetramethyl	0.91	0.80	0.55	blue	blue
	BAW	*15% acetic acid*	*Forestal*		
*Kampferol***					
5,4′-dimethyl	0.81	0.04	0.84	yellow	yellow
5,7,4′-trimethyl	0.87	0.08	0.91	yellow	yellow
3,5,7,4′-tetramethyl	0.93	0.32	0.97	blue	blue
*Quercetin****					
3,5,7,3′-tetramethyl	0.83	0.19	—	blue	yellow-green
5,7,3′,4′-tetramethyl	0.75	0.05	—	yellow	deep yellow
3,5,7,3′,4′-pentamethyl	0.85	0.20	—	blue	blue

* From NORDSTRÖM AND SWAIN[5].
** From HARBORNE[54].
*** From HERMANN[55].

TABLE VI

R_F VALUES OF FLAVANONES AND ISOFLAVONES

Flavonoid	*R_F values in*			
	Butanol–27% acetic acid (1:1)	*BAW (4:1:5)*	*Water*	*30% acetic acid*
*Flavanone aglycones**				
7,8,3′,4′-tetraOH**	0.72	—	—	0.55
7-OMe,8,3′,4′-triOH	0.76	—	—	0.63
8-OMe,7,3′,4′-triOH	0.82	—	—	0.65
7,8,4′-triOH**	0.81	—	—	0.62
6,7,3′,4′-tetraOH	0.73	—	—	0.46
7,3′,4′-triOH (butin)***	0.84	—	0.20	0.64
5,7,3′,4′-tetraOH (eriodictyol)***	0.85	—	—	0.56
5,7,4′-triOH (naringenin)***	0.69	0.89	0.16	0.66
7-OMe,5,4′-diOH (sakuranetin)	—	0.90	0.09	0.65
7,4′-diOMe,5-OH	—	0.91	0.06	0.66
5,7,3′-triOH,4′-OMe (homoeriodictyol)***	—	0.88	0.14	0.67
5,7,4′-triOH,3′-OMe (hesperitin)***	—	0.89	0.11	0.67
*Flavanone glycosides****				
Naringenin 7-rhamnoglucoside	—	0.59	0.62	0.87
Naringenin 7-glucoside	—	0.64	0.44	0.80
Sakuranetin 7-glucoside	—	0.69	0.48	0.81
Hesperitin 7-rhamnoglucoside	—	0.48	0.50	0.85
Hesperitin 7-glucoside	—	0.60	0.33	0.79
Flavanonols				
3,5,7,4′-tetraOH (dihydrokampferol)	—	0.87	0.30	0.72
3,5,7,3′,4′-pentaOH (dihydroquercetin)	0.86	0.78	0.28	0.67
Dihydrokampferol 7-glucoside***	—	0.57	0.64	0.84
Flavan				
3,5,7,3′,4′-pentaOH (*d*-catechin)	0.69	0.64	0.31	0.64
Isoflavone aglycones				
7,4′-diOH (daidzein)**	—	0.92	0.08	0.62
7-OMe,4′-OH (formononetin)**	—	0.94	0.07	0.67
7-OH,3′,4′-O_2CH_2 (ψ-baptigenin)**	—	0.94	0.07	0.66
5,7,4′-triOH (genistein)	—	0.94	0.04	0.59
7-OMe,5,4′-diOH (prunetin)	—	0.94	0.03	0.67
5,7,4′-triOH,8-OMe	—	0.91	0.11	0.70
5,7,8,4′-tetraOMe	—	0.93	0.29	0.84
5,7,4′-triOH,6-OMe (tectirigenin)	—	0.90	0.09	0.70
5,7,4′-triOH,6,3′,5′-triOMe (irigenin)	—	0.91	0.16	0.79
5,6,7,3′,4′,5′-hexaOH (irigenol)	—	0.54	0.02	0.33
Pomiferin	—	0.94	0.00	0.06
Osajin	—	0.95	0.00	0.00
Isoflavone glucosides				
Genistein 7-glucoside (genistin)	—	0.67	0.25	0.75
Tectirigenin 7-glucoside (tectiridin)	—	0.67	0.38	0.83
Irigenin 7-glucoside (iridin)	—	0.66	0.48	0.87

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* From HARBORNE AND GEISSMAN[9] and HARBORNE[54].

** Colour in U.V. with ammonia: light blue.

*** Colour in U.V. with ammonia: light yellow or yellow-green (the other compounds in this table have feeble colours or are colourless under these conditions).

are not available. The compounds that have been examined (Table V) can be separated by the usual solvent systems[54, 55].

Flavanones and isoflavones

The R_F values of a number of flavanones, isoflavones and other related substances are shown in Table VI. These compounds have a much more restricted distribution in the plant kingdom than flavones. Most of the 16 or so naturally occurring isoflavones are included. The collection of the R_F values of flavanones is also fairly comprehensive. Water and dilute acetic acid are very useful solvent systems with these compounds.

The R_F values of two flavanonols and a common catechin are included in Table VI for purposes of comparison. A typical monomeric flavan-3,4-diol, leucocyanidin, has R_F 0.52 in BAW, R_F 0.50 in water and other compounds of this type have very similar R_F values[25]. The positions of these latter compounds are located on paper by spraying with 3% *p*-toluenesulphonic acid in ethanol. Red or orange yellow spots develop after heating at 103° for 5 minutes[25].

TABLE VII

R_F VALUES OF CHALCONES AND AURONES*

Structure	*R_F values in*				
	*Butanol–27% acetic acid (1:1)***		*Phenol*	*Water*	*30% acetic acid*
*Chalcone aglycones****					
2′,4′,3,4-tetraOH (butein)	0.83	0.83	0.66	0.01	0.19
2′,4′,5′,3,4-pentaOH (stillopsidin)	0.65	—	—	0.01	—
2′,3′,4′,3,4-pentaOH (okanin)	—	0.56	—	—	0.08
Glucosides					
Butein 4′-glucoside (coreopsin)	0.67	0.56	0.64	0.05	0.43
Stillopsidin 4′-glucoside (stillopsin)	0.47	—	—	0.02	—
Okanin 4′-glucoside (marein)	—	0.38	—	—	0.22
Aurone aglycones					
6,3′,4′-triOH (sulphuretin)	0.87	0.80	0.70	0.01	0.19
4,6,3′,4′-tetraOH (aureusidin)	0.66	0.57	0.29	0.01	0.10
6,3′,4′-triOH, 7-OMe (leptosidin)	0.80	0.76	0.80	0.01	0.19
6,7,3′,4′-tetraOH (maritimetin)	—	0.53	—	—	0.10
Glucosides					
Sulphuretin 6-glucoside (sulphurein)	0.60	0.49	0.69	0.03	0.41
Aureusidin 6-glucoside (aureusin)	0.36	0.28	0.35	0.01	0.16
Aureusidin 4-glucoside (cernuoside)	0.56	0.49	0.45	0.02	0.25
Leptosidin 6-glucoside (leptosin)	0.55	0.51	0.73	0.03	0.33
Maritimetin 6-glucoside (maritimein)	—	0.42	—	—	0.21

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* From GEISSMAN[17], GEISSMAN AND HARBORNE[57], GEISSMAN, HARBORNE AND SEIKEL[52] and HARBORNE AND GEISSMAN[9].

** In the first column R_F's were measured from front of the spots; in the second column, R_F's were measured from centre of the spots.

*** All chalcones and aurones are yellow in visible light and yellow, orange or brown in ultraviolet light.

Chalcones and aurones

The R_F values of many of the naturally occurring chalcones and aurones[17, 52] and some of their methylated derivatives[9, 57] are collected in Tables VII and VIII. In these series, the glycosides have lower R_F values in BAW than the corresponding aglycones. The amount of this decrease in R_F value depends on the number and position of substitution of the sugar residues. For example sulphuretin 6-diglucoside (R_F 0.21 in BAW, 6:1:2)[58] has a lower R_F than the 6-monoglucoside (R_F 0.49; *cf.* sulphuretin R_F 0.80) and the 4- and 6-monoglucosides of aureusidin are chromatographically different (Table VII).

TABLE VIII

R_F VALUES OF METHYLATED CHALCONES AND AURONES

Compound	R_F values in				Colours	
	Butanol-water triphosphate paper	*BAW*	*30% acetic acid*	*Butanol ammonia*	*U.V.*	*U.V./NH$_3$*
*Butein**						
4',3,4-trimethyl	0.86	—	—	—	brown	brown
2',4',4-trimethyl	0.83	—	—	—	yellow-green	green
2',3,4-trimethyl	0.38	—	—	—	olive	yellow-green
2',4',3-trimethyl	0.68	—	—	—	yellow-green	yellow
2',4',3,4-tetramethyl	0.92	—	—	—	yellow-green	yellow-green
*Sulphuretin**						
3',4'-dimethyl	0.25	—	—	—	yellow-green	green
4',6-dimethyl	0.93	—	—	—	yellow	yellow-green
3',6-dimethyl	0.54	—	—	—	yellow	orange
3',4',6-trimethyl	0.80	—	—	—	green	green
*Aureusidin***						
4,3',4'-trimethyl	—	0.77	0.11	0.11	blue-green	blue-green
4,6,3'-trimethyl	—	0.77	0.11	0.33	yellow	orange
4,6,4'-trimethyl	—	0.81	0.22	0.59	blue-green	blue-green
6,3',4'-trimethyl	—	0.79	0.14	0.13	blue-green	blue-green
4,6,3',4'-tetramethyl	—	0.80	0.17	—	green	green
*Maritimetin****						
7,3',4'-trimethyl	—	0.83	0.18	—	yellow-green	orange
6,3',4'-trimethyl	—	0.83	0.20	—	green	brown
6-monomethyl	—	0.69	0.18	—	yellow	red-green
6,7,3',4'-tetramethyl	—	0.82	0.18	—	yellow-green	yellow-green
6,7-dimethyl	—	0.78	0.19	—	yellow	red

* From Nordström and Swain[58].
** From Geissman and Harborne[57].
*** From Harborne and Geissman[9].

The R_F values in 30% acetic acid of 17 synthetic aurones have been recorded by Roubalova[59].

3. CHROMATOGRAPHIC BEHAVIOUR AND CHEMICAL STRUCTURE

The R_F values of the majority of flavonoids are directly related to their chemical structure. This correlation between chromatographic behaviour and structure was first discovered by BATE-SMITH AND WESTALL[13]. These authors plotted

$$R_M \left(= \log\left(\frac{1}{R_F} - 1\right)\right)$$

values in BAW against the number of hydroxyl and glucosyl substituents. They found that a straight line relationship exists between the R_M values of flavonoids and the numbers of their hydroxyl and sugar substituents. The few compounds with irregular chromatographic behaviour also have unusual structural features. ROBERTS[10, 23, 24] and his co-workers have shown that this relationship is observed with R_F values in aqueous solvents as well. SIMPSON AND GARDEN[60] and SHAW AND SIMPSON[61] have shown that the chromatographic behaviour of some simple hydroxy and methoxy flavones is dependent on their degree of chelation. ROUX AND EVELYN[25] have found that R_M value is directly related to average molecular weight in the case of tannins having flavan units in their structure.

Relationships between chromatographic behaviour and structure of known flavonoids are illustrated in Tables II–VIII. The five main conclusions can be summarised as follows:

(1) The R_F values are sufficiently characteristic to make them of considerable value in the identification of flavonoids. There are many reports of the successful separation of related compounds. Two examples may be quoted, namely the separation of the optical antipodes of the tea catechins by ROBERTS AND WOOD[23] and the separation of the isomeric di- and trimethyl ethers of apigenin and luteolin by NORDSTRÖM AND SWAIN[5]. The number of pairs of flavonoids which are difficult or impossible to separate on paper are comparatively few and are, in any case, substances of very similar chemical structures. Examples are rhamnetin and isorhamnetin[46] (Table II), quercetin and aureusidin[26] and the 3-glucoside and 3-galactoside of kampferol[24].

(2) An increase in the number of hydroxyl groups substituted in the flavonoid molecule lowers the R_F value in both alcoholic and aqueous solvent systems[10, 13]. This result is to be expected in the solvent BAW but not in water, in which solvent an increase in hydroxyl groups might be expected to raise rather than lower the R_F value[10]. ROUX AND EVELYN[25] have shown that this generalisation only applies to phenolic hydroxyl groups and additional aliphatic hydroxyl groups do, in fact, increase the R_F value in water. These authors have also pointed out that *o*-hydroxyl groups reduce the R_F value in BAW more than two hydroxyl groups which are not adjacent.

(3) Methylation of their hydroxyl groups usually increases the mobility of flavones in all solvent systems. The rise in R_F value for every hydroxyl group that is methylated is much less than the fall in R_F value caused by the introduction of a hydroxyl group[13]. As may be expected the effect on R_F value of a chelated hydroxyl group is less than

that of a free hydroxyl group. Similarly, the methylation of a chelated hydroxyl group (*e.g.* that in the 5-position) will alter the R_F value more than methylation elsewhere in the molecule (compare the R_F's of quercetin and its 5-, 7- and 3'-methyl ethers, Table II).

(4) Glycosidation lowers the R_F value in BAW but increases it in water[10, 13]. This effect applies to all classes of flavonoid, but is best illustrated by reference to the flavonol glycosides (Table III). Increases in the number of sugar residues progressively reduce the mobility of flavonols in BAW and raise their mobility in water (compare the R_F values of 3-mono, 3-di and 3-triglucosides of kampferol and quercetin). The position of substitution of the sugar residues is an important factor in determining the R_F value of a glycoside. Kampferol and quercetin 7-monoglucosides for example have zero R_F values in water. On the other hand flavonol 3,7-triglycosides have higher R_F values in water than related 3-triglycosides.

(5) The R_F values in water of all planar flavonoid molecules are zero[10, 62]; for example flavonol and flavone aglycones, chalcones, aurones and anthocyanidins. In contrast flavonoids in which the two benzene rings are not in the same plane have positive R_F values in water; *e.g.* flavan-3-ols (catechins), flavan-3,4-diols, flavanones, flavanonols, dihydro-chalcones, dihydro-aurones and most flavonol glycosides. This generalisation has been established by the observations of ROUX[62] and ROBERTS, CARTWRIGHT AND WOOD[24]. ROUX AND EVELYN[25] have also pointed out that the introduction of planarity reduces the R_F values of flavonoids in alcoholic solvents, *e.g.* BAW, or *sec.*-butanol. Thus flavanonols (*e.g.* dihydroquercetin, R_F 0.78) have higher R_F values than the corresponding flavonols (*e.g.* quercetin, R_F 0.64).

4. THE CHROMATOGRAPHIC SURVEY OF FLAVONOIDS IN PLANTS

Flavonoids are widely distributed in foodstuffs[63], have pharmacological interest (*e.g.*[64]) and are related to the natural tannins[65]. Many surveys of plant material have been carried out in order to study the taxonomic distribution of these pigments. Previous methods of detecting flavones in plants were based on simple colour tests, many of which were of doubtful validity when used on crude extracts. Chromatographic methods have the advantages that they separate the flavones from impurities, reveal mixtures of flavones and provide an accurate and rapid means of provisional identification, based on R_F values and colour reactions. The application of paper chromatography to this field has therefore been most fruitful.

In most survey work, concentrated plant extracts are applied to paper chromatograms, which are developed in one, or more frequently, two dimensions. In addition, or alternatively, acid-hydrolysed extracts are examined in the same way for the presence of aglycones. Flavonoid spots on paper chromatograms are unlikely to be confused with those of other plant pigments or other naturally occurring substances (*e.g.* simple phenols, alkaloids, etc.). They are quite distinct from those of other plant colouring matters, such as the carotenoids, chlorophylls or anthraquinones[66]. The anthocyanins are readily distinguished from flavones because of their highly characteristic colours in visible light. Within the group of flavonoid compounds, it is the flavones, flavonols and anthochlor pigments which are most easily detected in chromatographic surveys. The presence of flavanones and isoflavones may be missed unless special chromogenic sprays are used.

The R_F values and colour reactions of glycosides on two-dimensional chromatograms and of aglycones on a "Forestal" one-dimensional chromatogram enable the experienced worker to identify provisionally many of the flavonoids present in a plant extract. It is generally recognised that detailed chemical analysis (infrared and ultraviolet spectroscopy, melting point, etc.) is a necessary part of complete identification. Even the glycoside of a well-known flavonol, *e.g.* quercetin, cannot be identified solely on the basis of chromatographic comparison with authentic glycosides.

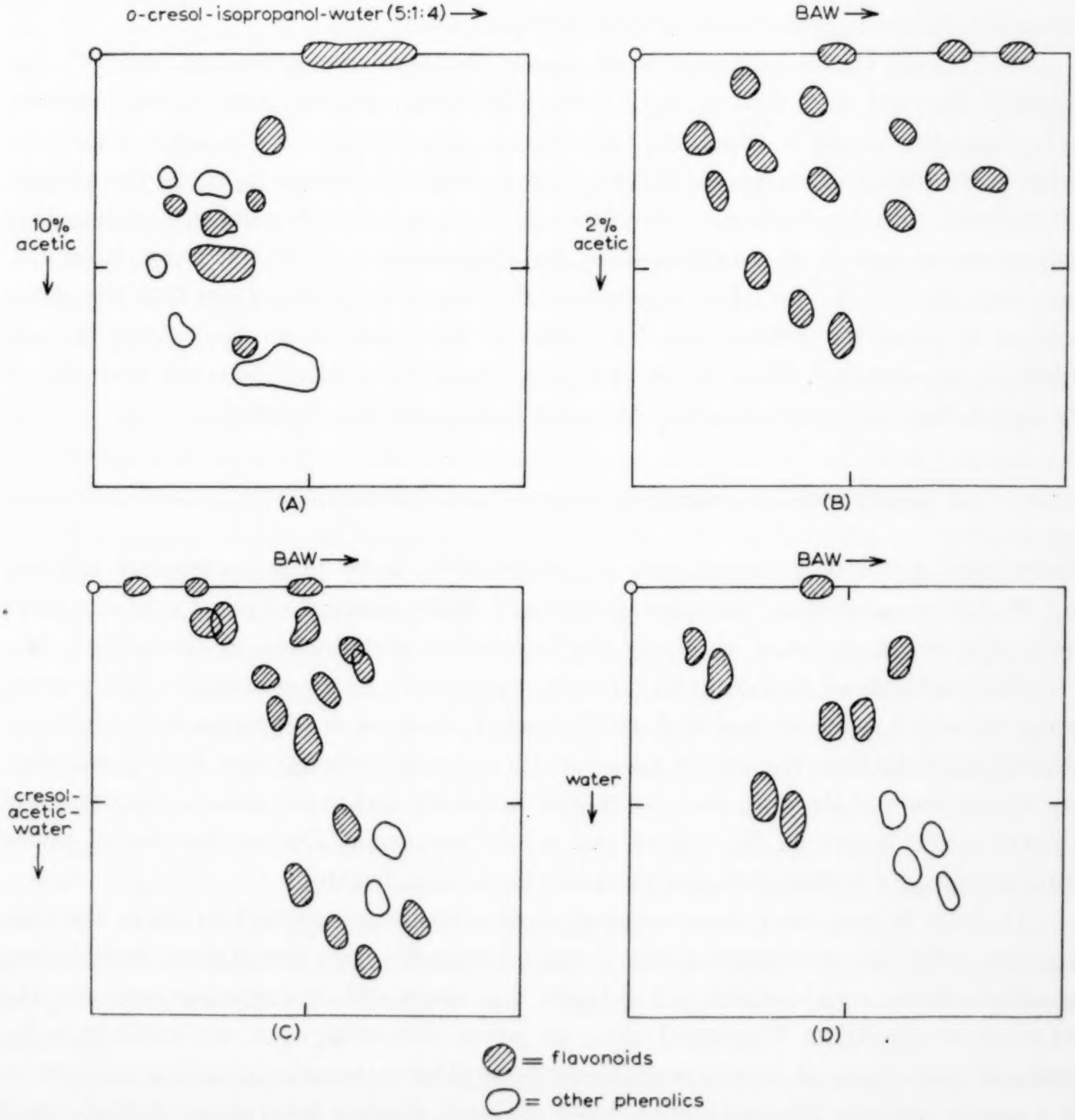

Fig. 2. Typical two-dimensional chromatograms of flavonoids present in plant extracts. (A) Leaves of *Triticum vulgare*[69], (B) Leaves of tea (flavonols only)[24], (C) Flowers of *Antirrhinum majus* (genotype PPMMYY)[6] and (D) Flowers of tuberous *Solanums*[54].

Chromatographic surveys are nevertheless an extremely useful way in which to study the occurrence of both known and unknown substances within a particular genus or family. The distribution of flavonoids in the leaves[4] and petals[3, 41] of vascular plants has been examined by paper chromatography. In more detailed surveys of groups of related plants, the complex patterns of flavonoid spots on two-dimensional chromatograms (see Fig. 2) have been used to study these relationships. Recent surveys of the heartwood extracts of *Pinus*[67] and *Prunus*[68] species, of the leaves of *Triticum* species, allied genera[69] and *Camellia* species[70], and of the tubers and flowers of *Solanum* species[54, 71] have all been carried out by means of paper chromatography.

The detection of flavonoids by two-dimensional chromatography has played an important part in recent biochemical-genetical studies of *Antirrhinum majus*[6] and *Dahlia variabilis*[72]. In this laboratory, the occurrence of flavones related to anthocyanins has been investigated using these and other methods[45, 53, 54]. Paper chromatography is the best way of examining the petals or leaves of plant progenies for variations in their flavone content.

Another use of paper chromatography is in the preparation of medicines of vegetable origin[73]. Spurious samples of plant material may be rejected on the basis of chromatographic examination of the flavonoids and other compounds present. Finally, paper chromatography has been used to investigate the metabolic fate of flavonoids when fed to rats and humans[74, 75].

5. SEPARATION AND PURIFICATION BY CHROMATOGRAPHY

Chromatography is a well recognised method for separating flavonoids present in plant extracts. Indeed for small scale work, paper partition chromatography is the method of choice. It is simple, effective and rapid. A high degree of resolution can be achieved without much experience. On the other hand, for large scale separation, chromatography has not supplanted the time-honoured procedures of lead precipitation, fractional crystallisation or the more recent development of counter-current distribution[76]. Chromatography by adsorption on columns of magnesol or cellulose works well with simple mixtures of flavones.The method has, however, failed to separate some complex mixtures of flavonoids[5, 49, 58]. The development of new adsorbents such as polyamide resin[41] may alter this picture.

The method of separating plant flavonoids by paper chromatography is as follows. It consists simply of banding concentrated plant extracts on sheets of thick Whatman filter paper, developing with a suitable solvent (*e.g.* BAW) and excising, eluting and rechromatographing the individual bands which have separated. Further details are given elsewhere[5, 6, 9, 53]. Fig. 3 shows how the flavonol glycosides of *Primula sinensis* flowers separate on paper. The method is particularly useful in providing a means of isolating minor components and those compounds with unusual solubility properties, which might be missed in conventional isolation procedures.

Pure, crystalline flavonoids are readily obtained from plant extracts after separation on paper. There are however some plant constituents which will not separate on paper. NORDSTRÖM AND SWAIN[5] failed to separate naringenin and apigenin glycosides in an extract of a mauve variety[5] and chalcone and aurone glycosides in yellow varieties[58] of *Dahlia variabilis*. They assumed that their difficulties were due to the formation of "complexes" between the flavanones (or chalcones) and the other flavonoids in these plant extracts. The author has noted similar difficulties in obtaining luteolin 7-glucoside in a pure form from partially hydrolysed extracts of parsley seed[52].

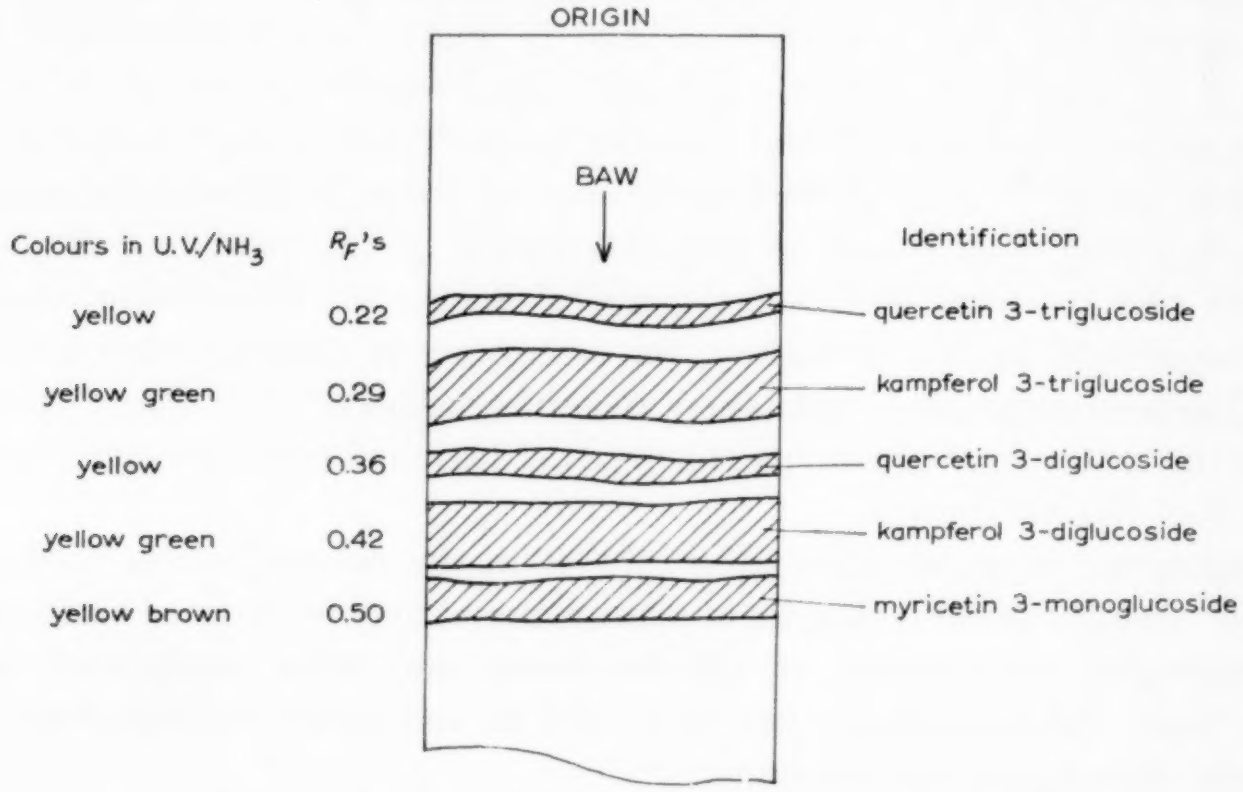
ORIGIN
BAW
Colours in U.V./NH₃
R_F's
Identification
yellow
0.22
quercetin 3-triglucoside
yellow green
0.29
kampferol 3-triglucoside
yellow
0.36
quercetin 3-diglucoside
yellow green
0.42
kampferol 3-diglucoside
yellow brown
0.50
myricetin 3-monoglucoside

Fig. 3. Chromatographic separation of flavonols of white flowers of *Primula sinensis* (genotype "beetle") on Whatman No. 3 filter paper.

Paper partition chromatography is very useful for determining the amount of separation that has been obtained by other purification procedures (*i.e.* column chromatography). It has also shown that material isolated by other extraction procedures as a single substance may contain more than one compound. For example, NYSTRÖM, HOWARD AND WENDER[77] separated two new flavonol glycosides from a supposedly pure sample of xanthorhamnin (rhamnetin 3-rhamninoside). By using 36 sheets of Whatman 3 MM paper and *n*-butanol–chloroform–acetic acid–water (4:1:1:1, by vol.) for development, they obtained 20, 80 and 10 mg of the three flavonoids from 300 mg of commercial material. In another case, a sample of "lotoflavin", which was thought to be 5,7,2′,4′-tetrahydroxy-flavone, was found on chromatographic analysis to be a mixture of kampferol and quercetin[78].

The separation of flavonoids by chromatography on magnesol was introduced and developed by GAGE, WENDER[7,8] and their associates. They isolated kampferol, quercetin and their glycosides from a number of fruit extracts (*i.e.* apricots[79], grapes[80], black-currants[81], strawberries[82] and whortleberries[83]). Other absorbents have been used for separating flavonoids. The flavones of *Spartium junceum* flowers were separated on a cellulose column by elution with water-saturated butanol by SPADA AND CAMERONI[50]. The isoflavone, biochanin A, was isolated from clover by chromatography on celite 545[39]. The tea catechins were separated on columns of silica gel by BRADFIELD AND PENNEY[38]. NEU[42] has separated synthetic mixtures of chalcones and flavanones on columns of polyamide resin. Finally, use has been made of chromatography on alumina or magnesol for purifying intermediates or products in chemical syntheses involving flavonoids (*e.g.*[54, 84]).

6. THE CHROMATOGRAPHIC IDENTIFICATION OF FLAVONOIDS

As mentioned in the introduction, paper chromatography plays an essential role in the identification of flavonoids on a micro-scale. The combination of this technique with that of ultraviolet spectroscopy has now been used in a large number of investigations for the elucidation of the structure of unknown compounds. The methods can be applied to as little as 1 mg of unknown substance[5] and many pigments have been identified without being isolated on a macro-scale[9]. These methods were first described by NORDSTRÖM AND SWAIN[5] and have been used and developed by numerous other investigators (*e.g.*[9, 10, 24]). The determination of the structure of flavonoid glycosides involves first the identification of the algycone, and then the determination of the nature, position and number of sugar residues. The application of paper chromatography to these two aspects will be considered separately.

Aglycone identification

In typical procedures[5, 52] the pure unknown glycoside (approx. 1 mg) is hydrolysed for 1 hour with normal mineral acid and the aglycone is extracted into ether or ethyl acetate. A portion of the concentrated extract is chromatographed and co-chromatographed with markers in at least three solvent systems (*e.g.* BAW, Forestal and phenol). A second portion is purified by paper chromatography for spectral examination[17]. All commonly found flavonoid aglycones can be distinguished satisfactorily by these methods. If the aglycone is a new one its chromatographic behaviour may suggest the way in which its structure differs from that of known compounds. Isolation of larger quantities for more detailed chemical analysis is then usually necessary. A further portion of the aglycone extract may be subjected to alkaline degradation and the simpler products (phenols and phenolic acids) identified by paper chromatographic means[78, 85]. Quercetin, for example, gives protocatechuic acid and phloroglucinol, both of which can be detected on paper, after separation in BAW, with diazotised *p*-nitroaniline[78]. The information obtained in this way is useful whether the aglycone under examination is a known or new compound.

One difficulty in this work is that some glycosides are peculiarly resistant to acid hydrolysis. At the same time, some aglycones, noticeably myricetin, are slowly destroyed by prolonged heating in acid solution. These factors may explain why some flavonoid glycosides do not give any aglycone, even after prolonged hydrolysis. Examples are a quercetin glycoside present in *Antirrhinum majus*[26], a kampferol derivative in *Freesia* flowers, apigenin glycosides of *Streptocarpus* garden hybrids[54], and flavone-like compounds in oil flax[86]. Other glycosides, *e.g.* those occurring in *Polygonum orientale*[87], are resistant to hydrolysis but do yield some aglycone.

Chromatographic methods are particularly applicable to the identification of flavonoid aglycones in cultivated plants. When complex mixtures of pigments occur and the analysis of individual pigments is not practicable or when single plants have to be tested for their flavonoid content, considerable reliance has to be placed on identifying the aglycone mixtures by paper chromatography. Such identifications can be confirmed by cutting out the separated aglycone spots from chromatograms, eluting and identifying by spectral methods. Solvents containing mineral acid should be avoided in this work as Bate-Smith[4] has found that flavonols undergo some change on chromatograms run in the Forestal solvent. Fig. 4 indicates the kind of separation that can be achieved when working with mixtures of aglycones. It shows a one-dimensional chromatogram of the hydrolysed extracts of flowers of *Verbena* garden hybrids[54]. Reference may also be made to *Antirrhinum majus*[6]. Mixtures of aglycones from this plant were separated and identified by two-dimensional chromatography.

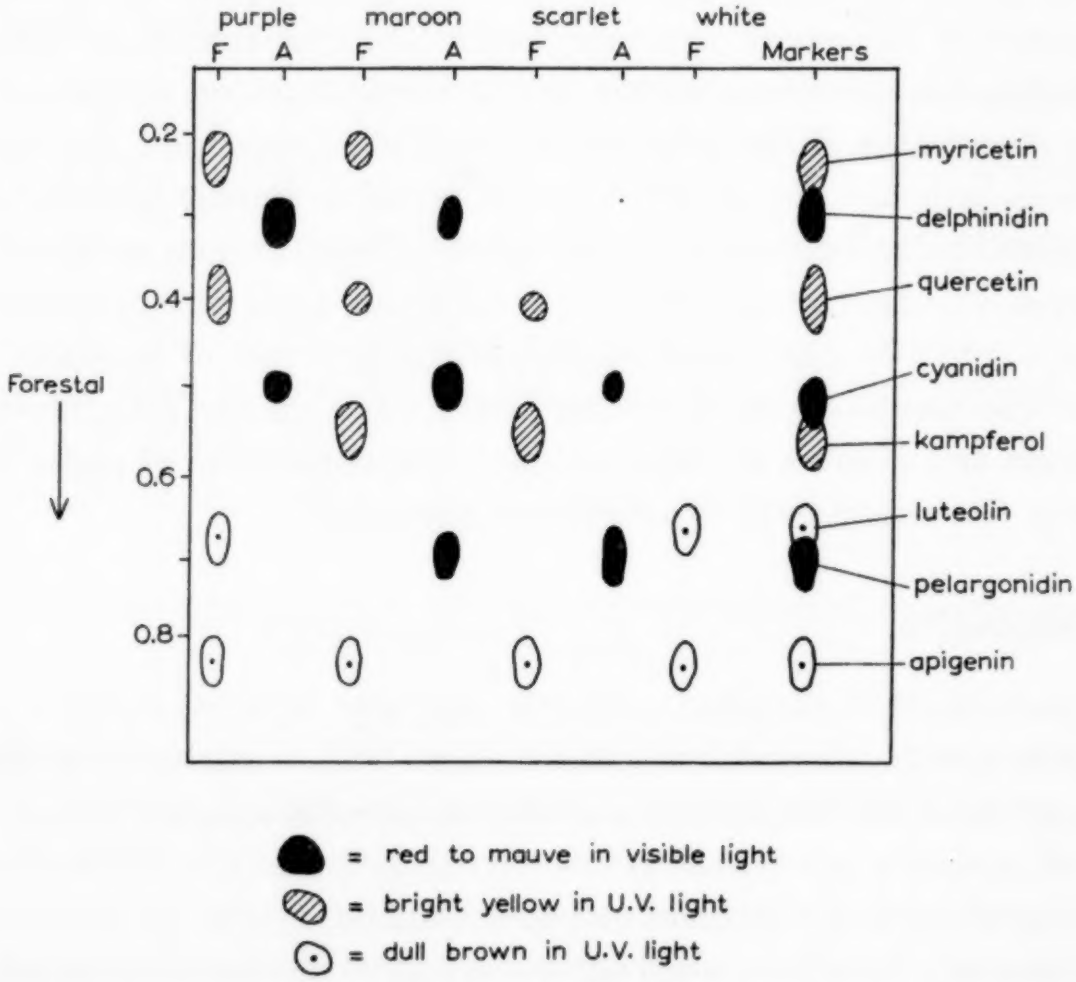
purple
maroon
scarlet
white
F A F A F A F Markers
0.2
0.4
0.6
0.8
Forestal
myricetin
delphinidin
quercetin
cyanidin
kampferol
luteolin
pelargonidin
apigenin
● = red to mauve in visible light
▨ = bright yellow in U.V. light
⊙ = dull brown in U.V. light

Fig. 4. One-dimensional chromatogram of flavonoid aglycones of colour mutants of *Verbena* garden hydrids; F = flavone extract (ethyl acetate), A = anthocyanidin extract (amyl alcohol).

Glycoside determination

Paper chromatography is a most valuable technique for identifying the glycosidic residues of flavonoids. Plant glycosides are often isolated in hydrated form and characterisation by classical methods (elementary analyses, m.p. etc.) may not be reliable. This particularly applies to glycosides containing two or more sugar residues, which may be difficult to purify by recrystallisation. The ways in which chromatography is useful in this work can be enumerated as follows.

(1) The chromatographic behaviour of an unknown glycoside, particularly in water as solvent, is a useful indication of the number and position of its sugar residues. A comparison of its R_F values with those of known glycosides of the same aglycone (see *e.g.* Tables III and IV) will show whether it is a new glycoside or not. One method of confirming that a particular glycoside occurs in a plant extract is to add the substance it is suspected to be to the extract. The spot of this substance on a two-dimensional chromatogram of the mixture is intensified if the two compounds are the same.

(2) The products of controlled acid hydrolysis of di- and triglycosides may be identified by paper chromatography. For example, the 3-rhamnogalactoside and 7-monorhamnoside of kampferol were formed during the hydrolysis of robinin (kampferol 3-rhamnogalactosido-7-rhamnoside)[54]. Likewise kampferol and quercetin 3-triglucosides yield the corresponding 3-diglucosides. Unlike the situation in the anthocyanin series[11], the method does not work well with flavonol 3-diglycosides. Rutin, quercetin 3-rhamnoglucoside, for example only gives traces of the 3-monoglucoside during hydrolysis[54]. On the other hand, flavone and flavanone 7-monoglucosides are easily prepared from the corresponding 7-apiosylglucosides and 7-rhamnoglucosides by partial acid hydrolysis[22, 37].

(3) The sugars produced by acid hydrolysis of flavonoid glycosides are identified by paper chromatography. Suitable procedures are described adequately elsewhere[88]. In purifying glycosides by chromatography for sugar determinations, free sugars can be removed by running a chromatogram in acetone–water (1:3), water or 15% acetic acid.

(4) Paper chromatography may also be useful when the sugar:aglycone ratios of flavonoid glycosides have to be determined. The amounts of aglycone produced by acid hydrolysis are most easily measured by spectrophotometry. The sugars are then separated by paper chromatography, developed with aniline hydrogen phthalate, eluted separately and the concentration of colour is measured spectrophotometrically[89, 90]. Alternatively the sugars are eluted off the paper and then developed by reaction with anthrone[77]. Sugar estimations can also be made without first hydrolysing the glycoside, *i.e.* by reaction with anthrone or a resorcinol–sulphuric acid reagent[55, 91].

(5) The position of substitution of sugar residues is determined in the following way. The glycoside is fully methylated. Acid hydrolysis then produces a methylated derivative which has free hydroxyl groups in positions that were originally substituted with sugar residues[5, 9]. Paper chromatography and ultraviolet spectroscopy are used for distinguishing and identifying these derivatives on a micro-scale. The isomeric derivatives of a particular aglycone, *e.g.* apigenin (Table V), have different colour reactions, R_F values or spectral maxima (*cf.*[5]). The requisite data are, however, still only available for a limited number of flavonoid compounds (Tables V and VIII).

CONCLUSION

The review will conclude with a summary of the advantages and disadvantages of chromatographic methods of identification. It is appropriate to do this as the value of chromatography for reducing the time and labour required for identifying unknown flavonoids is still not fully appreciated. On the other hand, the necessity for checking chromatographic identifications by means of other methods is not always realised.

The first advantage is that chromatography provides a rapid method of identification using milligram instead of gram amounts of material. Secondly, the R_F or R_M value is a new physical constant, which is comparable with the melting point as a means of identification. Thirdly, chromatography will separate the complex mixtures of pigments, which are frequently found in cultivated plants. Fourthly, it provides the first reliable method of rapidly surveying plant material for the presence of flavonoids.

The limitations of chromatography should also be borne in mind. Thus chromatography must always be used in conjunction with other appropriate analytical procedures. There is also a definite limit to its resolving power since some plant components which behave as single chromatographic entities have been shown to be mixtures. Finally, while it is of outstanding value with simple pigments, chromatography is of more limited value in the identification of flavonoids of complex structure (*e.g.* bisflavonoids, rotenoids, isoprenyl flavones, etc.).

The main future development in this field will undoubtedly be in the identification of more naturally occurring pigments, since all the flavonoid constituents have been identified in only a few plant species. Such information will increase our knowledge of the biosynthetic processes and functions of these pigments in the plant cell. For identification purposes, the measurement of R_F values of more reference compounds is needed. New solvent systems are unlikely to oust those now in use, but the discovery of some new adsorbents for column chromatography would be welcome. Finally, since chromatography can be used to detect traces of material, this technique should be of outstanding value in metabolic studies of flavonoids in plants.

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PAPER ELECTROPHORESIS AND PAPER CHROMATOGRAPHY OF PHENOLIC COMPOUNDS

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The introduction of new analytical techniques, and in particular chromatography, has been largely responsible for the rapid increase, during the last decade, of our knowledge of the biochemistry of phenolic compounds.

The literature on the chromatography of phenolic compounds is extensive but good reviews of developments, up to 1956, are available (BRAY AND THORPE[1]; CLARKE AND NORD[2]; LEDERER AND LEDERER[3]). Amongst the more recent publications in this field is a comprehensive study by REIO[4] of the identification and separation of phenols by paper chromatography.

By comparison with chromatographic methods, the paper electrophoresis of phenolic compounds has been neglected. MICHL[5] has investigated the electrophoresis of borate complexes of *o*-dihydric phenols and COULSON AND EVANS[6] have used borate buffers for their studies with phenolic carboxylic acids and other phenolic derivatives. SHIMOMURA[7] and BERBALK AND SCHIER[8] have also reported electrophoretic studies with phenols.

The present enquiry was undertaken in connection with work on the biosynthesis of phenolic glycosides by plants (PRIDHAM[9]) in an endeavour to supplement existing techniques for the identification and separation of small amounts of phenolic materials. Particular attention has been paid to the use of molybdates as reagents both for the detection and the separation of *o*-dihydroxy compounds by paper electrophoresis and paper chromatography, and the electrophoretic behaviour of phenolic compounds in several different buffer systems has been examined.

EXPERIMENTAL

Molybdate complex formation

The ability of various phenolic compounds to form coloured molybdate complexes was tested by spotting their solutions on to strips of Whatman No. 3 paper which had previously been dipped in a 0.1 *M* sodium molybdate solution, blotted with filter paper, then air-dried. The rapid development of an intense orange-brown colour was taken as a positive indication of complex formation.

Paper electrophoresis

The paper electrophoresis apparatus used for these experiments was built according to a design kindly provided by Dr. D. GROSS of Tate & Lyle, Ltd., and the individual runs on Whatman No. 3 paper were continued for 1 ½–2 hours at 22 V/cm. Diazotised *p*-nitroaniline solution (as prepared by SWAIN[10]) followed by *N* NaOH solution, was used to locate the phenols on the paper, and all mobilities were calculated as M_{SA} values, that is, relative to the mobilities of salicylic acid (*o*-hydroxybenzoic acid), 2,3,4,6-tetra-O-methyl-D-glucose being used as a zero marker. *p*-Anisidine hydrochloride (HOUGH, JONES AND WADMAN[11]) was used to locate this carbohydrate derivative. When using alkaline buffers it was necessary to expose the zero marker strips to moist hydrogen chloride gas prior to spraying with the *p*-anisidine hydrochloride reagent. The "absolute mobility" of *o*-hydroxybenzoic acid in each buffer was also calculated.

Buffers

0.2 *M* acetate (pH 5.2)
$8.1 \cdot 10^{-3}$ *M* molybdate (pH 5.2)*
0.2 *M* phosphate (pH 7.2)
0.2 *M* borate (pH 10.0)
0.1 *M* glycine (pH 10.0)

Paper partition chromatography

The descending method of chromatography was adopted and the solvent systems used were butan-1-ol–ethanol–water (4:1:5 v/v; organic layer) and ethyl acetate–acetic acid–water (9:2:2 v/v). Plain and molybdate-treated Whatman No. 3 papers were used for these experiments and diazotised *p*-nitroaniline/NaOH as the spray reagent. The rates of movement of the phenols were recorded as R_F values.

RESULTS AND DISCUSSION

Spot tests with approximately one hundred phenolic compounds using the molybdate-treated paper showed that the presence of an *o*-dihydroxy or *ene-diol* grouping was essential for the formation of a coloured molybdate complex. The test substances, in addition to simple phenols, included phenolic carboxylic acids and nitro- and aminophenols. Particular attention was paid to phenols having hydroxyl groups *ortho* to other substituents. Tests were also negative with a large number of carbohydrate derivatives, but L-ascorbic acid, an *ene-diol* compound, gave a strong positive reaction.

RILEY[12] has used phosphomolybdic acid followed by ammonia as an unspecific chromatographic spray reagent to detect polyhydric phenols and recently COULSON AND EVANS[6] have used a molybdate reagent to detect *o*-dihydric phenols. With this latter method, however, the chromatograms have to be sprayed with three different solutions. On the basis of the present work it would appear that sodium molybdate alone is a highly specific reagent for *o*-dihydroxy compounds and in this laboratory an aqueous solution (0.1 *M*) has been used successfully as a chromatographic spray reagent. The immediate appearance of orange-brown spots after spraying with this reagent is a clear indication of *o*-dihydric phenols. Other phenolic compounds may react slowly to give coloured spots but this only occurs several hours after spraying.

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* $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1% w/v) in 0.002 *N* H_2SO_4.

Paper electrophoresis

The results of the paper electrophoresis study are given in Table I, which shows the relative mobilities of a number of phenolic compounds using various buffer systems. In some instances the compounds "tailed" badly (perhaps due to oxidation) and this has been indicated in the table. Using an acetate buffer (pH 5.2) it was observed that

TABLE I

RELATIVE ELECTROPHORETIC MOBILITIES OF PHENOLIC COMPOUNDS EXPRESSED AS M_{SA} VALUES

$$M_{SA} = \frac{\text{distance moved by phenolic compound}}{\text{distance moved by } o\text{-hydroxybenzoic acid}}$$

Compound	*M_{SA} values*				
	Acetate pH 5.2	*Molybdate pH 5.2*	*Phosphate pH 7.2*	*Borate pH 10.0*	*Glycine pH 10.0*
Quinol	0	0	0.01	**	**
Catechol*	0	1.07	0.01	0.67	0.51
Resorcinol	0	0	0.01	0.35	0.44
Phloroglucinol	0	0	0.01	1.27	1.05
Pyrogallol*	0	0.98**	0.01	0.75**	**
Hydroxyquinol*	0	1.07	1.25**	1.20**	1.16**
3,4-Dihydroxyphenylalanine*	0	0.81	0.01	0.72	**
o-Hydroxybenzoic acid	1.00	1.00	1.00	1.00	1.00
m-Hydroxybenzoic acid	0.81	0.79	0.85	1.05	0.95
p-Hydroxybenzoic acid	0.64	0.70	0.77	1.21	1.10
Protocatechuic acid*	0.50	1.00	0.67	1.19	1.02
Gallic acid*	0.45	1.03	0.59	1.20	0.93
p-Coumaric acid	0.34	0.44	0.58	0.98	0.86
Caffeic acid*	0.20	0.79	0.50	1.04	1.05
Ferulic acid	0.21	0.21	0.42	0.87	0.77
Chlorogenic acid*	0.33	1.03	0.44	0.83	0.74
p-Hydroxybenzaldehyde	0	0	0.27**	0.84**	0.79**
Vanillin	0	0	0.27	0.79	0.76
Syringaldehyde	0	0	0.21	0.71	0.72
Umbelliferone	0	0	0.04	0.76	0.63
4-Methyl-umbelliferone	0	0	0	0.52	0.50
4-Methyl-aesculetin*	0	0.61	0.11	0.48	0.27
Arbutin	0	0	0	0.22	0.09
Aesculin	0	0	0.26	0.51	0.24
Phloridzin	0	0	0.08	0.42	0.14
Catechin*	0	0.23	0	0.65	0.63
Quercetin*	0	0.05	0	0.21	0
Myricetin*	0	0	0	0.18	0
Luteolin*	0	0	0	0.11	0
Kaempherol	0	0	0	0.12	0
Genistein	0	0	0.01	0.25	0.16
Irigenin	0	0	0.04	0.52	0.35
"Absolute mobilities" of *o*-hydroxybenzoic acid (cm/h/1200 V)	8.8	8.8	8.0	9.7	10.8

* *o*-Dihydroxy compounds.
** Compounds decomposed or "tailed".

phenolic groups were insufficiently ionised to allow migration but compounds possessing carboxyl groups moved rapidly towards the anode. The flavonoids, which were examined, were virtually insoluble in this buffer. With regard to the phenolic carboxylic acids, there appeared to be no simple correlation between their mass/charge ratios and relative mobilities. The relative mobilities observed using the molybdate buffer (pH 5.2) were, in many cases, similar to those obtained with the acetate buffers. Compounds possessing *o*-dihydroxy groupings, however, formed coloured complexes immediately the molybdate buffer was applied to the paper and these, with the exception of the flavonoid derivatives, migrated rapidly towards the anode. The flavonoids and flavonoid–molybdate complexes (with the exception of catechin and possibly quercetin) again appeared to be insoluble in this acidic solution.

The effect of complex formation on mobility is well illustrated by a comparison of the M_{SA} values of catechol and resorcinol in the two acidic buffers.

At pH 7.2 (phosphate buffer) the mobilities of the phenolic carboxylic acids were again, in general, much greater than those of compounds possessing only phenolic groups, but at this pH some movement of many of the latter compounds was observed. Their relative mobilities, however, were normally small and under these conditions it must be assumed that the phenolic hydroxyl groups were only weakly dissociated.

In more strongly alkaline solutions some movement was noted with the majority of phenolic compounds which were examined. In general, with both glycine and borate (pH 10.0) buffers an increase in molecular weight with constant charge retarded the rate of movement. The latter was clearly shown by the phenolic aldehydes where the relative mobilities were in the following order: *p*-hydroxybenzaldehyde > vanillin > syringaldehyde. The high mobilities of the monohydric phenolic aldehydes and also the coumarin derivatives, in alkaline buffers, were rather surprising and suggested that, possibly, these compounds in some way reacted with the basic components of the buffers.

In borate buffer, evidence of complex formation by the *o*-dihydroxy compounds was again obtained, although the results were not so marked as with the molybdate buffer. The borate complexes with *o*-dihydric phenols are presumably similar to those formed with glycols. The behaviour of the flavonoid and trihydroxybenzene derivatives was unpredictable.

At these higher pH values the phenolic hydroxyl groups were highly dissociated and had a marked influence on mobilities. This is apparent when the rates of movement of the phenolic acids in alkaline and acidic buffers are compared. In the majority of cases the mobilities of these compounds were greater in alkaline solutions than in acid because of the increased charge due to hydroxyl dissociation. The behaviour of the isomeric monohydroxybenzoic acids in borate buffer is particularly interesting. Their relative mobilities were in the following order: *para* > *meta* > *ortho*, this being a reversal of the mobilities found with buffers of pH 5.2 and 7.2. At these lower pH values the mobilities of the monohydroxybenzoic acids bore a positive relationship to the dissociation constants, but it is difficult to explain the movement of these compounds in borate buffer.

In glycine buffer the order of the mobilities of the monohydroxybenzoic acids differed from those in borate and all other buffers which were examined, the M_{SA} values for the *ortho, meta* and *para* derivatives being 1.00, 0.95 and 1.10 respectively. The differences between the mobilities of these three acids in the glycine buffer were small but nevertheless definite.

In general it would appear that the rate of movement of phenolic compounds on electrophoretograms is dependent on three main factors; the molecular weight, the degree of dissociation of functional groups, and the ability to form charged complexes with the components of the electrolyte solutions. The latter, in the case of borate and molybdate buffers is governed by the stereochemistry of the phenol. Other factors, such as hydration and distribution of charge, probably also influence mobilities.

This investigation has shown that paper electrophoresis is a valuable tool for the examination of mixtures of phenolic compounds such as those commonly found in extracts of plant tissues. The components of these mixtures which are often present only in trace quantities can normally be identified accurately by comparing their electrophoretic behaviours with those of known specimens, in several different buffers. It is advisable to make a direct comparison of authentic and unidentified specimens on the same electrophoretogram, rather than a comparison of mobilities with those values recorded in the literature. When authentic specimens for comparison are not available, or a new phenolic compound has been isolated, paper electrophoresis can assist in structural determinations. For example, carboxyl and *o*-dihydroxy groupings can be detected quite readily. Paper electrophoresis of relatively high molecular weight compounds, such as phenolic glycosides and flavonoids, is of limited value as their mobilities are usually small, unless strongly ionised groups are present. Some separation of these compounds can be achieved by the use of buffers with high pH values provided the compounds are not alkali-labile.

Paper partition chromatography

WACHTMEISTER[13] and SWAIN[10] have used borate buffered paper for the chromatography of phenolic compounds and, in particular, to detect *o*-dihydric phenols. The movement of these latter compounds was retarded by borate owing to complex formation and similar results have now been obtained using molybdate-treated paper (Table II). The advantage of using molybdate-treated paper is that *o*-dihydroxy compounds form coloured complexes which generally move as discrete spots and spray reagents are therefore not required. Borate complexes are colourless. It was observed that the retardation of movement of *o*-dihydroxy compounds by molybdate was, in general, more marked in the neutral solvent than in the acidic solvent. Using sodium borate-treated papers alone it may be difficult to distinguish between *o*-dihydroxy compounds and phenolic carboxylic acids in neutral solution, as the latter compounds could be retarded owing to the formation of sodium salts. However, by using molybdate this difficulty is overcome as only the *o*-dihydric phenols give the coloured complexes.

Paper chromatography using molybdate-treated paper would appear to offer a

TABLE II

PAPER CHROMATOGRAPHY OF PHENOLIC COMPOUNDS USING PLAIN AND MOLYBDATE-TREATED PAPERS WITH ACIDIC AND NEUTRAL SOLVENTS

Compound	*R_F values*			
	Butan-1-ol–ethanol–water solvent		*Ethyl acetate–acetic acid–water solvent*	
	Plain paper	*Molybdate-treated paper*	*Plain paper*	*Molybdate-treated paper*
Phenol	0.94	0.97	0.96	0.91
Quinol	0.87	0.91	0.87	0.68**
Catechol*	0.88	0.38	0.88	0.60
Resorcinol	0.88	0.90	0.88	0.78
Phloroglucinol	0.76	0.76	0.74	0.84
Pyrogallol*	0.77	0.02	0.79	0.25
Hydroxyquinol*	0.74	0.03	0.74	0.34
3,4-Dihydroxyphenylalanine*	0.08	0	0.19**	0.01
o-Hydroxybenzoic acid	0.13	0.15	0.17	0.29
m-Hydroxybenzoic acid	0.25	0.33	0.95	0.92
p-Hydroxybenzoic acid	0.22	0.26	0.94	0.92
Protocatechuic acid*	0.82	0.06	0.86	0.24
Gallic acid*	0.58**	0	0.65	0.11
p-Coumaric acid	0.36	0.36	0.92	0.81
Caffeic acid*	0.68	0.01	0.83	0.44
Ferulic acid	0.23	0.25	0.92	0.90
Chlorogenic acid*	0.19**	0	0.59	0.01
4-Methyl-aesculetin*	0.76	0.21	0.83	0.26
Catechin*	0.63**	0.15	0.68	0.33
Quercetin*	0.14**	0.03	0.84	0.49**
Genistein	0.71**	0.78**	0.98	0.98
Irigenin	0.59**	0.65**	0.97	0.98

* *o*-Dihydroxy compounds.
** Compounds gave elongated spots.

rapid method to distinguish between *o*-dihydroxy compounds, and other phenols, and in conjunction with the electrophoretic techniques, which have been described, a great deal can be done towards the elucidation of the complete structures of unidentified phenolic compounds.

ACKNOWLEDGEMENTS

The electrophoresis apparatus was built with the aid of a grant from the Royal Society. The author wishes to thank Professor E. J. BOURNE for his advice and encouragement, and Dr. J. W. T. SEAKINS and Dr. T. SWAIN for kindly providing some of the specimens.

SUMMARY

An aqueous solution of sodium molybdate has been used as a specific reagent for the detection of phenolic compounds with *o*-dihydroxy groupings.

The paper electrophoretic behaviour of phenolic compounds has been studied using a series of different buffers with pH values ranging from 5.2 to 10.0. *o*-Dihydroxy compounds can readily be detected by the use of ammonium molybdate buffer, and

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other structural features can be revealed by the choice of suitable electrolyte solutions.

Paper partition chromatography of phenolic compounds using molybdate-treated paper is described.

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PINAKRYPTOLGELB — EIN GEEIGNETES REAGENS ZUM NACHWEIS DER ARYLSULFONSÄUREN AN CHROMATOGRAMMEN

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Zur Sichtbarmachung der Flecken der Arylsulfonsäuren an Papierchromatogrammen wurden bisher nur wenige Methoden empfohlen. Die bequemste und empfindlichste (0.01–1 µg) ist die Fluoreszenz im ultravioletten Licht, die zum Beispiel bei den Anthrachinon-[1] und Anthracensulfonsäuren mit Erfolg benutzt wurde. Für Naphthalinsulfonsäuren, die beim Bestrahlen mit der Quarzlampe nicht fluoreszieren, wurde die Bestrahlung mit einem Licht von bestimmter Wellenlänge (253 mµ) empfohlen[2]. Ein anderer Nachweis der Sulfonsäurenflecken wird durch Erhitzen des Chromatograms auf 200° im Trockenschrank durchgeführt[3]. Dabei karbonisiert das Papier an Stelle der Anionen- und Kationenflecken. Auf solche Weise können noch 5–20 µg Sulfonsäuren nachgewiesen werden; bei manchen Säuren, z.B. bei Anthrachinonsulfonsäuren, genügt die benutzte Temperatur aber nicht dazu die Zersetzung hervorzurufen. Ein weiterer Nachteil dieser Methode ist, dass der Nachweis nicht spezifisch ist, da in gleicher Weise verschiedenste anorganische und organische Anionen und Kationen reagieren. Auch der Nachweis der Sulfonsäuren mittels Säure–Basen-Indikatoren ist nicht spezifisch und genügend empfindlich[4].

Wir haben jetzt gefunden, dass zur Sichtbarmachung der Sulfonsäureflecken Pinakryptolgelb[5] geeignet ist. Dieses Reagens wurde schon früher zur Sichtbarmachung der höheren Alkylsulfate benutzt[6]. Mit Sulfonsäuren entstehen auf den Chromatogrammen charakteristisch gelb, orangefarben bis gelbbraun fluoreszierende Flecken auf hellgrünem Hintergrund. Durch längeres Bestrahlen werden sie dunkler. Bei geringster Konzentration der chromatographierten Verbindungen, die noch

TABELLE I

PAPIERCHROMATOGRAPHISCHE TRENNUNG UND IDENTIFIZIERUNG DER SULFONSÄUREN

Verbindung	R_F	*Erfassungsgrenze µg*	*Fluoreszenz*
Benzolsulfonsäure	0.74	10–20	gelborange
p-Toluolsulfonsäure	0.80	3–10	orangebraun
Xylolsulfonsäure	0.81	4–6	hellgelb
1-Naphthalinsulfonsäure	0.80	1–3	gelb, braunstichig
2-Naphthalinsulfonsäure	0.82	1–3	gelb
1,5-Naphthalindisulfonsäure	0.53	1–2	gelb
1,3,5-Naphthalintrisulfonsäure	0.31	0.5–2	gelb, braunstichig
1,3,5,7-Naphthalintetrasulfonsäure	0.15	0.5–1	intensiv gelb, braunstichig

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erfassbar ist, erscheinen die Sulfonsäuren erst nach bis 60 Minuten dauernder Bestrahlung und zwar direkt als dunkle Flecken. Der Nachweis ist empfindlich und die Erfassungsgrenzen sind aus der Tabelle I ersichtlich.

Neben den in der Tabelle I angeführten Sulfonsäuren untersuchten wir das Verhalten einer Reihe anderer Verbindungen, das in einigen Fällen charakteristisch war (Tabelle II). Die Anionen anorganischer Säuren erschienen als schwierig erkennbare helle Flecke. Eine Ausnahme bildeten die Bromide (schwacher dunkler Flecken) und Sulfite (intensive blauviolette Fluoreszenz). Werden die Sulfonsäuren als Natrium- oder Kaliumsalze an die Chromatogramme aufgetragen, erscheinen die Kationen als blaugraue Flecken erst nach längerem Bestrahlen.

TABELLE II

PAPIERCHROMATOGRAPHISCHE IDENTIFIZIERUNG VERSCHIEDENER VERBINDUNGEN

Verbindung	R_F	*Verhalten im UV-Licht nach dem Besprühen*
Natrium	0.20	blaugraue Fluoreszenz
Kalium	0.12	blaugraue Fluoreszenz
4-Nitrotoluol-2-sulfonsäure	0.84	intensiv gelbe Fluoreszenz
4-Aminotoluol-2-sulfonsäure	0.73	intensiv braunviolette, später braune Fluoreszenz
Sulfanilsäure	0.49	schwach gelbbraune Fluoreszenz
Phenolsulfonsäure	*	gelbe bis gelbbraune Fluoreszenz
1-Naphthalinkarbonsäure	0.77	gelbe, später braune Fluoreszenz
Laurinsäure	0.84	intensiv gelbe Fluoreszenz, die durch das Bestrahlen verschwindet
Taurin	—	kein fluoreszierender Flecken
1,4-Naphthylaminsulfonsäure	0.62	die ursprüngliche violette Fluoreszenz wird tiefer
1,4-Naphtholsulfonsäure	0.42	die ursprüngliche blauviolette Fluoreszenz wird tiefer und schlägt bald nach braun um
1-Anthrachinonsulfonsäure	0.72	charakteristische intensive Fluoreszenz der Anthrachinonsulfonsäuren (gelbe bis grüne) schlägt nach dem Besprühen nach gelbbraun um
2-Anthrachinonsulfonsäure	0.80	
1,5-Anthrachinondisulfonsäure	0.39	
1,6-Anthrachinondisulfonsäure	0.49	
1,7-Anthrachinondisulfonsäure	0.52	
1,8-Anthrachinondisulfonsäure	0.60	
2,6-Anthrachinondisulfonsäure	0.59	
2,7-Anthrachinondisulfonsäure	0.59	
1-Anthracensulfonsäure	0.78	die ursprüngliche blauviolette Fluoreszenz schlägt an den Ränden in braun um

* Diese Säure (Acidum phenolsulfonicum-Merck) zeigte 4 Flecke (R_F: 0.12; 0.35; 0.53; 0.71).

Die angeführten Verbindungen wurden im System *n*-Propanol–Ammoniak (2:1) auf Whatmanpapier No. 3 absteigend chromatographiert. Die Laufzeit war *ca.* 4 Stunden, wobei das Lösungsmittel eine Strecke von 25 cm durchlief. Das Chromatogram wurde bei 100° getrocknet, mit einer 0.05% wässrigen Pinakryptolgelblösung besprüht und sofort mit UV-Licht bestrahlt (Original Quarzlampe Hanau oder Phillora HPW 125). Durch Trocknen bei höherer Temperatur zeigen einige Verbindungen etwa geänderte Färbungen. Die Pinakryptolgelblösung wurde in braunen Flaschen aufbewahrt und war unbeschränkt haltbar.

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ZUSAMMENFASSUNG

Es wird eine empfindliche Methode beschrieben für die Identifizierung der Arylsulfonsäuren in Papierchromatographie. Das Papier wird mit Pinakryptolgelb besprüht und in UV-Licht untersucht.

SUMMARY

A sensitive method for the identification of arylsulphonic acids is described, in which paper chromatograms are sprayed with Pinakryptol Yellow and then examined under UV light.

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ORGANIC ACIDS IN A SELECTED DIALYSATE OF AIR PARTICULATE MATTER*

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During chromatographic studies of the air particulate matter from urban air, it became increasingly apparent that the variations in composition of the organic acid fraction, as well as the complexity of the air sample, made the interpretation of chromatograms unduly involved. While the method of CORCORAN[1] had more than adequate resolving power for aliphatic acids from C_1 to C_{10}[2], the absence of any detailed knowledge of the nature of the carboxylic acids in the air made the preparation of the sample for chromatography a trial and error process. To overcome this difficulty selective dialysis[3] was used so that a given air sample, after dialysis, could be chromatographed in stages. Since the more rapidly dialysing solutes have the lower molecular weights, each stage of the chromatographic sample increases progressively with respect to the molecular weight of its organic acid components. The present report is concerned with the application of such selective dialysis to five air particulate samples from the National Air Sampling Network of the Community Air Pollution Program. This study was confined to one sample of each of five selected cities, and to the two-hour dialysate of the sodium hydroxide extract of the organic acids of these samples.

PROCEDURE

An amount of the glass filter containing approximately 150 mg of air particulate matter was cut into rectangles having an area of approximately one-sixteenth square inch. The sample was placed in 100–150 ml of 0.05 *N* sodium hydroxide solution, and the mixture was shaken mechanically. In order to determine the time required for maximal recovery of the acids from the particulate, quantities of $H^{14}COOH$ of known specific activity as the sodium salt were added to particulate matter, and the amount of naturally occurring acid extracted was deduced by the isotope dilution principle. Since 5.5 hours was found to be sufficient time for such extraction, the extract was decanted through a filter of glass wool after this interval of time. 10 ml of the filtrate were placed in a cellophane dialyzing bag having a diameter, when round, of 19 mm. CRAIG's procedure of selective dialysis was followed, and the alkaline extract of the particulate, reduced by evaporation to a volume of 10 ml, was dialyzed against 100 ml of water. The dialysate collected after 2 hours was used for these studies with the exception that the material used in the isotope studies with aniline-^{14}C described below employed a 24-hour dialysate. Dialysates in all cases were taken to dryness under moving warm air. The dried residue (sodium salts of the organic acids) was dissolved in from 0.3 to 0.8 ml water, acidified to approximately pH 2 (or lower) with glycine buffer and/or 1–1 HCl. The acidified solution, having a volume between 0.5 and 1.0 ml was mixed with 0.6 to 1.2 g of silicic acid and stirred. This mixture was suspended in chloroform and placed on the top of a silica gel column of such mass that the combined weight of the silica mixed with the sample and used for the column was 3.0 g. Silica gel chromatography followed the procedure of CORCORAN[1] except that gradient elution[4] was employed and the aqueous phase was glycine buffer pH 2 exclusively. The first effluent chromatographic zone, a possible mixture of aliphatic acids having more than 3 carbons, was examined qualitatively by reacting these carboxylic acids with aniline-^{14}C. When the reaction mixture was chromatographed according to DE JONGE[5], radioautographs charted the chromatographic mobility of the resulting anilides.

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* Paper presented at the Symposium on Air Pollution at the 134th National Meeting of the American Chemical Society, September 7–12, 1958, Chicago, Ill.

Two samples of soot from burning propane and toluene, kindly furnished by Professor B. D. TEBBENS, University of California, were extracted, dialyzed and chromatographed according to the procedure described above for the air particulate matter.

RESULTS AND DISCUSSION

The data of Table I indicate that the specific activity of the chromatographically isolated H^{14}COOH does not decrease after 5.5 hours. Obviously, therefore, the concentration of inert, naturally occurring formic acid in the extract does not increase, and thereby dilute the introduced radioactive organic acid after 5.5 hours. This

TABLE I

SPECIFIC ACTIVITY OF INTRODUCED H^{14}COOH* AFTER SEVERAL PERIODS OF EXTRACTION OF AIR PARTICULATE MATTER WITH 0.05 N SODIUM HYDROXIDE

Time (h)	*Specific activity*
2**	1910 1903 1880
5.5**	1553 1618
24***	1565 1560 1750

* Specific activity, 12,940 cts/mmole.
** With mechanical shaking.
*** 6 h shaking, 12 h standing and 6 h shaking.

relatively simple extraction procedure for these acids argues against the use of more drastic methods involving heat for such extraction purposes.

Chromatograms from the 2-hour dialysates from the selected air samples appear in Fig. 1. It is clear that all the samples are different with respect to the concentration of organic acids in the 2-hour dialysate. The two that are most nearly alike are the Cincinnati and the Racine samples and even these differ with respect to two factors. The first of these factors is that the acidity of the first 44 ml of the effluent of the Racine sample indicates the presence of acid solutes while the corresponding portion

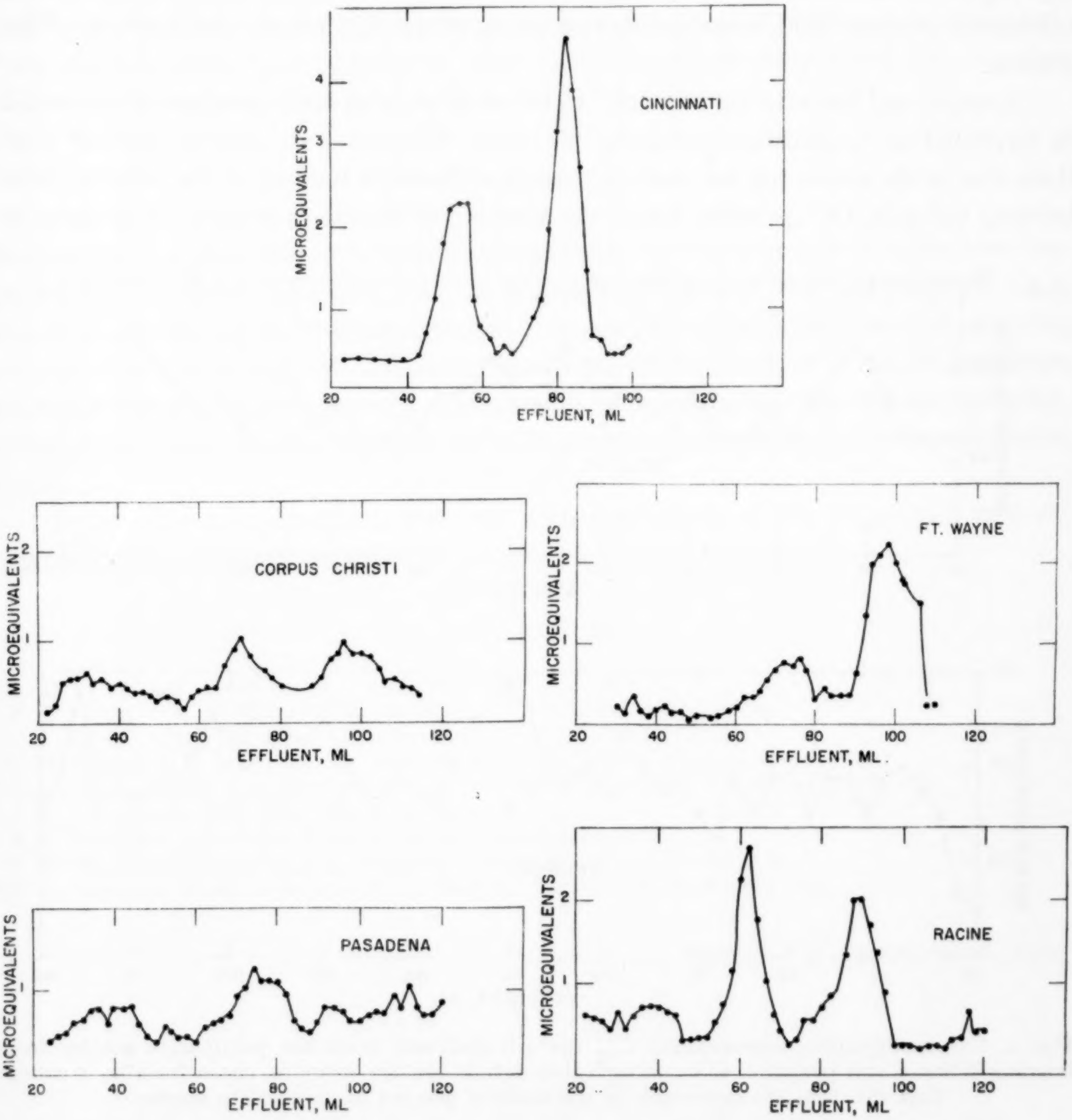

Fig. 1. Chromatographic representation of the acids emerging from silica gel columns from 2-h dialysates of 5 samples of air particulate matter. 3-g silica gel columns were employed with an aqueous phase of glycine buffer pH 2. The mobile phase consisted of butanol admixing with chloroform in a 500-ml mixing vessel. Plots express the data on the basis of 200 mg of air particulate matter. Samples are identified by the National Air Sampling Network as: Cincinnati, 12106; Corpus Christi, 12153, 12173, 12172; Fort Wayne, 12660; Pasadena, 15832; Racine, 12484.

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of the effluent from the Cincinnati air is apparently free of such solutes. The second factor is that, while the acidity between the 76th and 96th effluent ml is approximately 1.5 times that between fractions 44 and 62 for the Cincinnati particulate, the corresponding zones are nearly equal for the dialysate from Racine. The acidity residing within the 76th and 98th ml has been shown[2] to be that of formic acid by microdiffusion studies, while that of the zone between fractions 45 and 68 is acetic acid with a carboxylic acid contaminant.

On a weight basis, the formic acid concentration as determined by this method is 1.52 μg/m^3 for Cincinnati and 0.52 μg/m^3 for Racine. The impure acetic acid fraction, calculated as acetic acid, corresponds to 1.19 μg/m^3 for Cincinnati, and 0.58 μg/m^3 for Racine.

Acetate and formate are known[6,7] to occur in air, and their presence in air would be expected as combustion products. BUSKYE, WILDER AND HOBBS showed[3] that these two acids accounted for over 65% of the titratable acidity of the effluent from burning tobacco. On the other hand, the burning of simple fuels such as propane or toluene was evaluated through selective dialysis. Fig. 2 shows that the greater part of the titratable acidity of the selected dialysate does not reside in acetic and formic acids. There is the possibility that these acids are liberated in appreciable quantities, on the combustion of these fuels, but they are not adsorbed or otherwise bound by

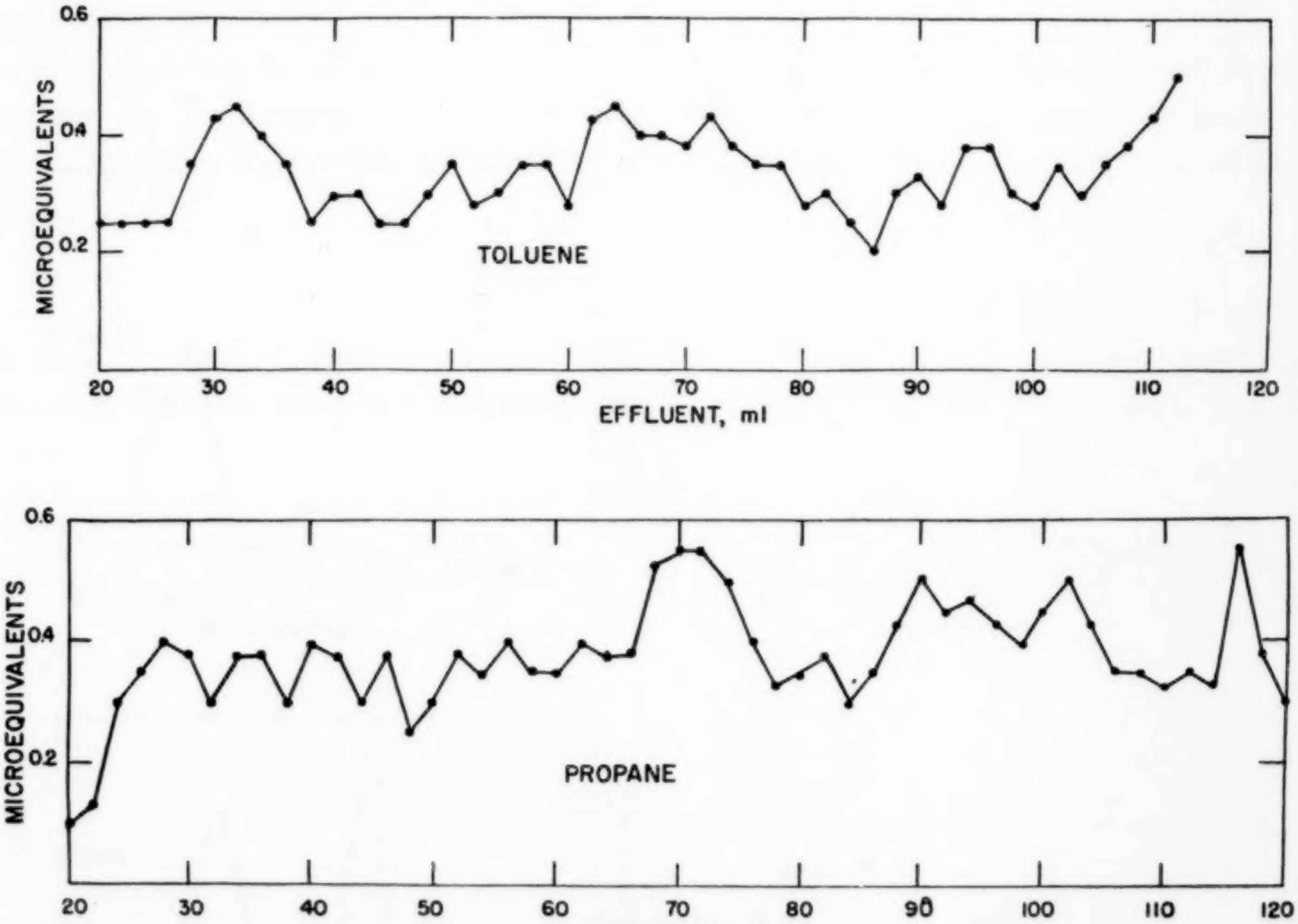

Fig. 2. Chromatographic representation of the 2-h dialysate from the particulate matter from burning toluene and propane. Chromatographic details are the same as those for Fig. 1 except that the data are expressed on the basis of 500 mg of particulate matter.

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the particulate constituting the soot. The basic difference between the simpler acid patterns (Cincinnati and Racine) as contrasted with the more complex (Corpus Christi, Fort Worth and Pasadena) could also reflect chemical interaction of the components of air as well as their source. Therefore it seemed worthwhile to critically evaluate the particulate collections from Racine and Cincinnati in order to decide the validity of the similarity suggested by the above data.

When pooled samples representing the acidity of the first 35 ml of the Racine and Pasadena samples were reacted with aniline-^{14}C, the radioautographs showed no distinct spots at the known R_F values for the anilides of aliphatic acids having from 3 to 10 carbons atoms. A single spot near the front appeared on the radioautograph from the described chromatographic zone of the Pasadena sample and the chemical nature of this anilide is being investigated.

SUMMARY

Because of the difficulty of interpreting partition chromatograms of acids from air particulate samples, selective dialysis through cellophane was investigated as a means of simplifying the elution patterns. Five samples of particulate matter collected on glass fiber filters, and two samples of soot from the combustion of simple fuels were analyzed. The procedure consisted of extraction, dialysis, and column chromatography, followed by paper chromatography of radioactive anilides of unresolved portions of eluate.

The results are presented, together with evaluation of the technique, and recommendations for further study.

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MORPHOLOGICAL IDENTIFICATION OF SOME ORGANIC ACIDS AS THE SODIUM SALTS

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In an investigation of the characterization of organic acids by quantitative microdiffusion[1], it was observed that a number of the acids gave visible characteristic crystal aggregates of the sodium salts as a result of the diffusion process. These crystals can serve to confirm the identity of the diffusing acid, as well as provide, by their speed of formation, a rough index of diffusion rate. The crystals were generally of moderate size (up to *ca.* 5 mm long), and well formed; the technique appears useful for growing crystals suitable for further crystallographic characterization. The addition of a drop of chloroform frees the crystals from the pellet.

The acids diffused from appropriate solutions in the outer well of a sealed Conway cell to a sodium hydroxide pellet in the inner well. As expected[1] characteristic crystal aggregates formed only in those cases where diffusion was most rapid.

PROCEDURE

Large samples (0.5 ml or more) of nonaqueous solutions were allowed to diffuse in 7.5 cm Conway cells (Microchemical Specialties Company), while the smaller and aqueous samples were run in 3 cm cells. The tight-fitting tops of the cells were sealed with petrolatum. An attempt was made to use sodium hydroxide chips and small samples on glass microscope slides with watch glass covers sealed with petrolatum, but was not successful, because in most cases the chips dissolved before crystals could form, or there was no crystal formation at all.

Photomicrographs were taken of significant samples by incident illumination, using a Galileo Model ZC microscope.

RESULTS AND DISCUSSION

The results for 2 % solutions are summarized in Table I. Characteristic crystal aggregate forms are shown in Figs. 1–7. It will be noted that the mixture of formic, propionic, and isovaleric acids gave crystals characteristic of isovaleric acid, while the mixture of valeric and isovaleric acids produced no crystals in two hours.

The morphology changed with the volume and concentration of the acids and the time. With large amounts of acid (0.5 ml or more) and high concentrations, the

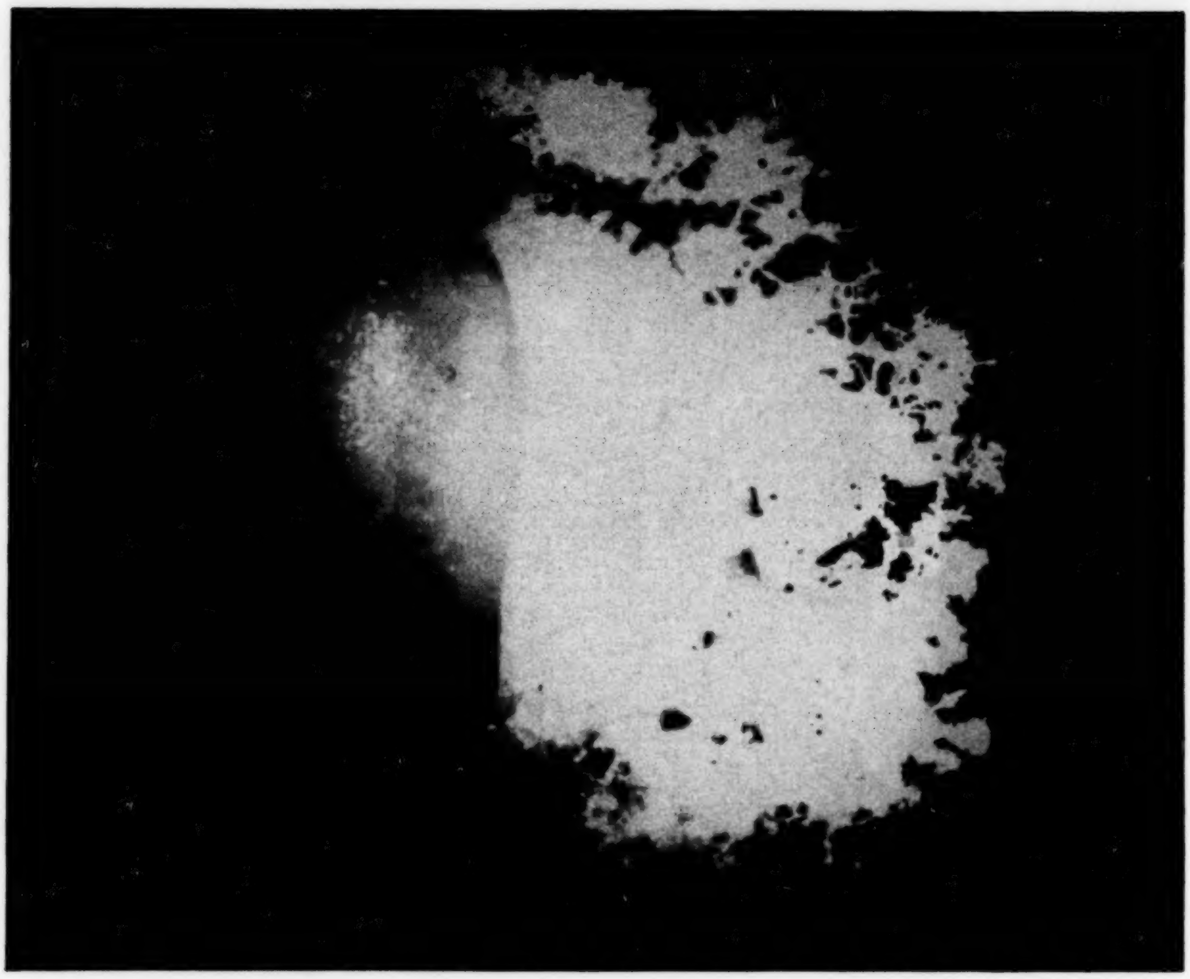

Fig. 1. Sodium formate crystals from diffusion of formic acid from cyclohexane. The sodium hydroxide pellet in each figure is approximately 5 mm in diameter.

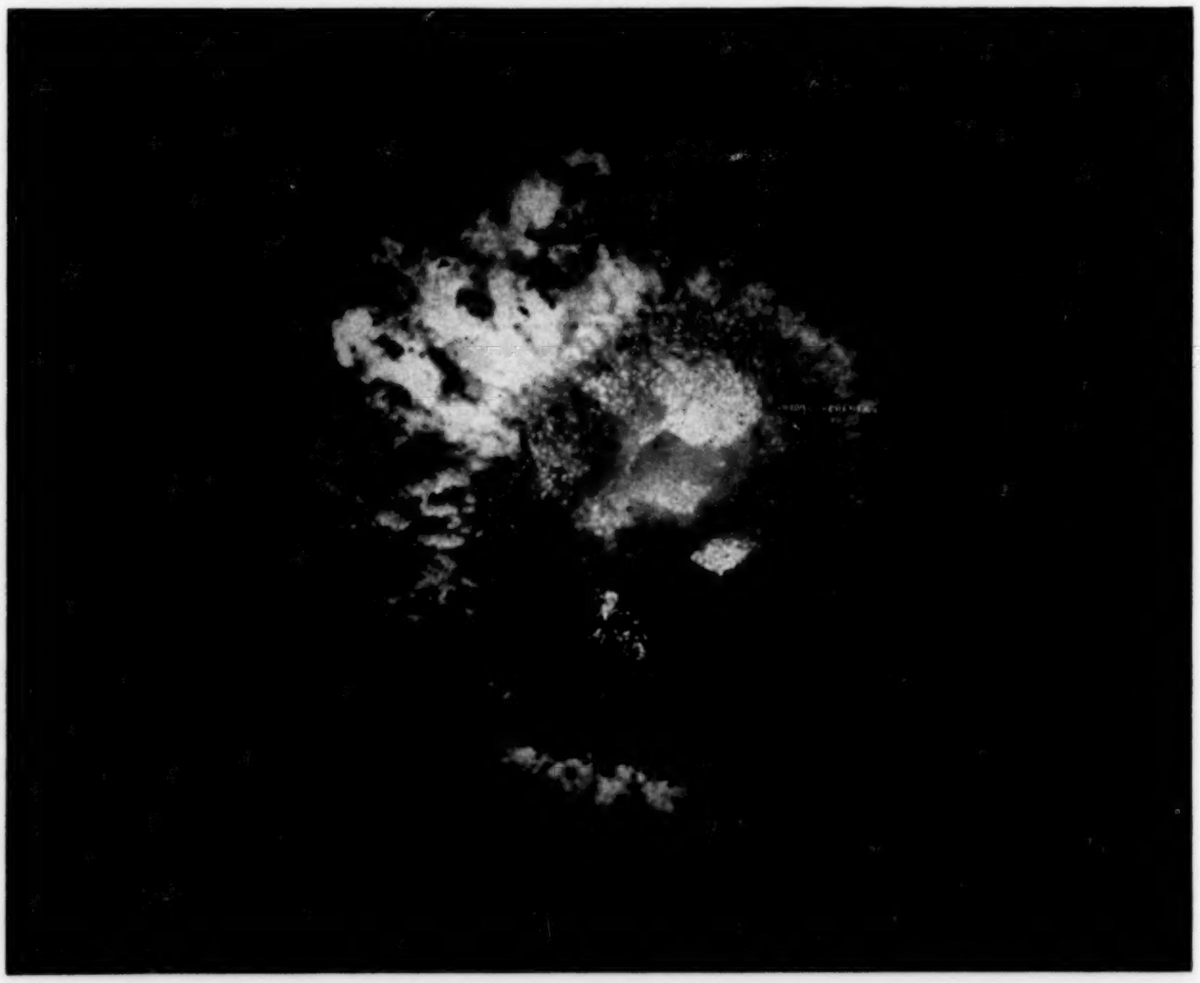

Fig. 2. Sodium formate crystals from diffusion of formic acid from benzene.

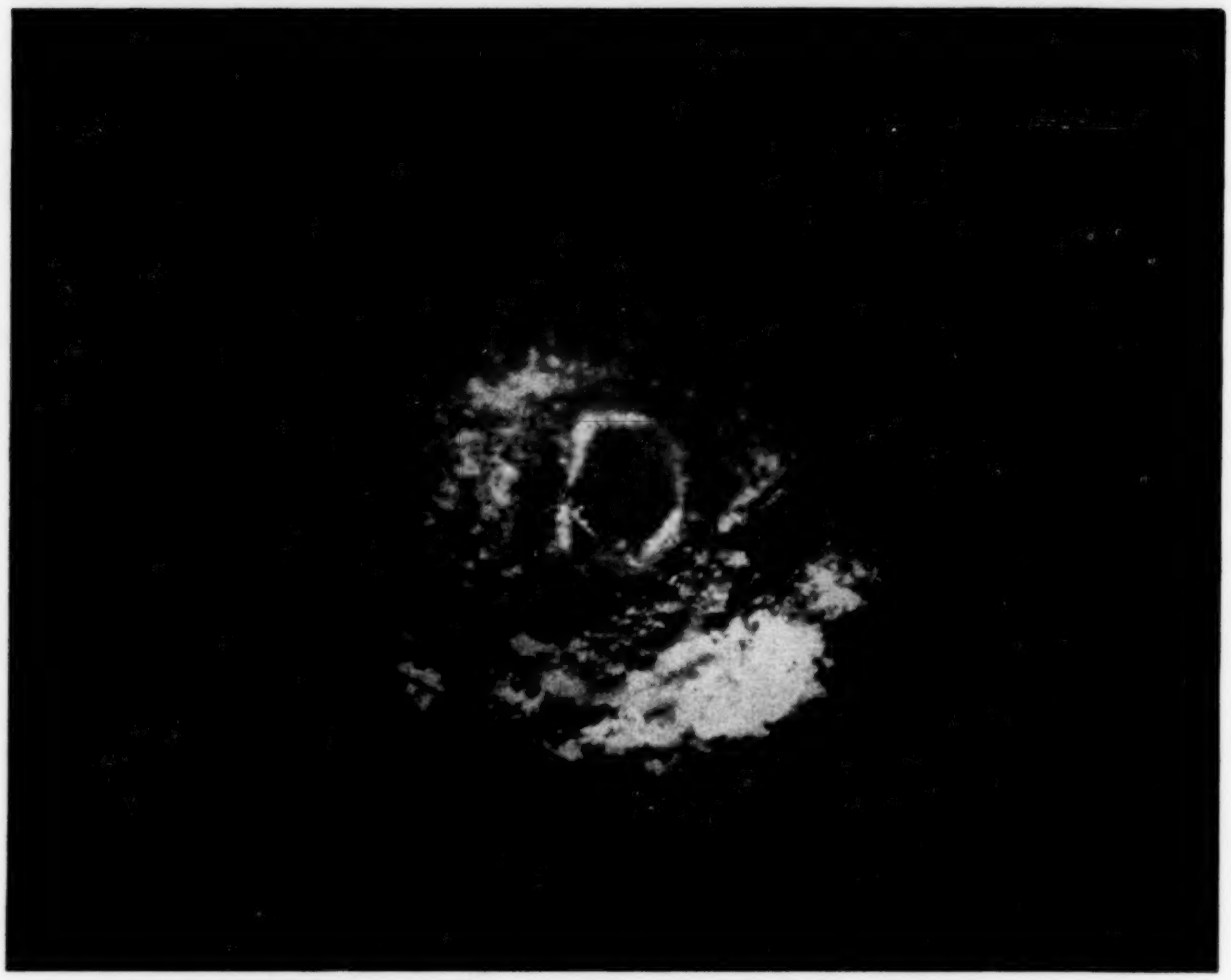

Fig. 3. Sodium acetate crystals from diffusion of acetic acid from cyclohexane.

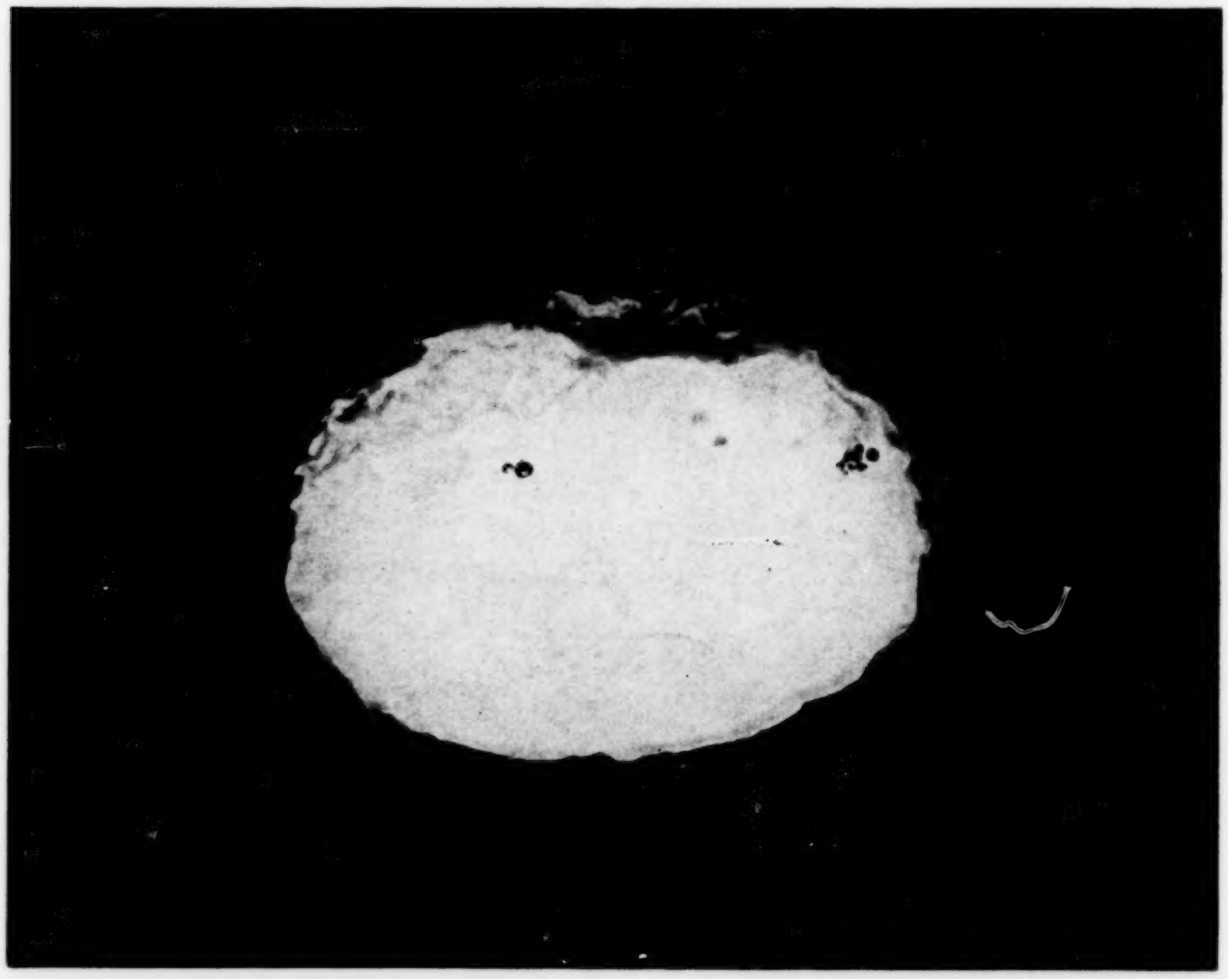

Fig. 4. Product of diffusion of acetic acid from benzene.

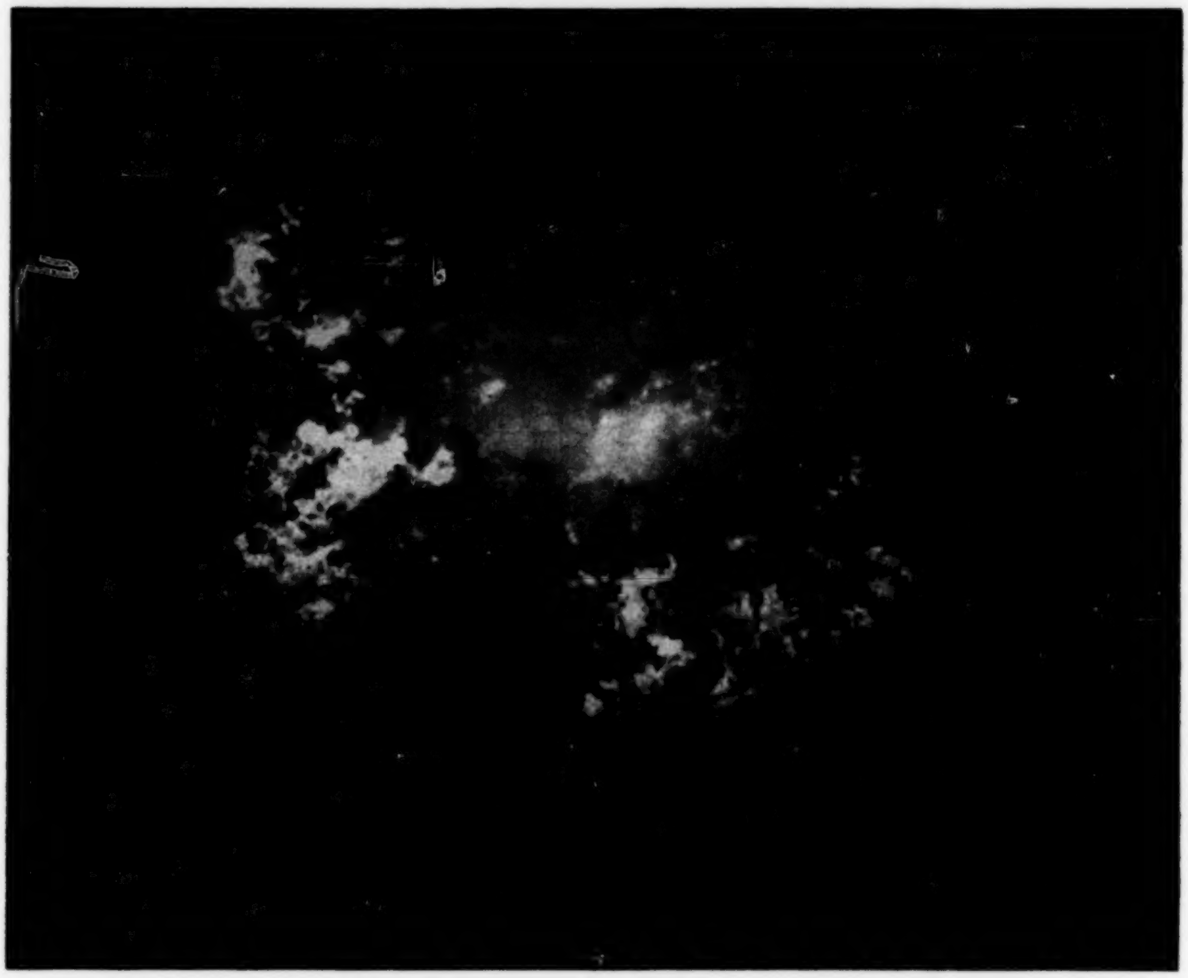

Fig. 5. Sodium isobutyrate crystals from diffusion of isobutyric acid from water.

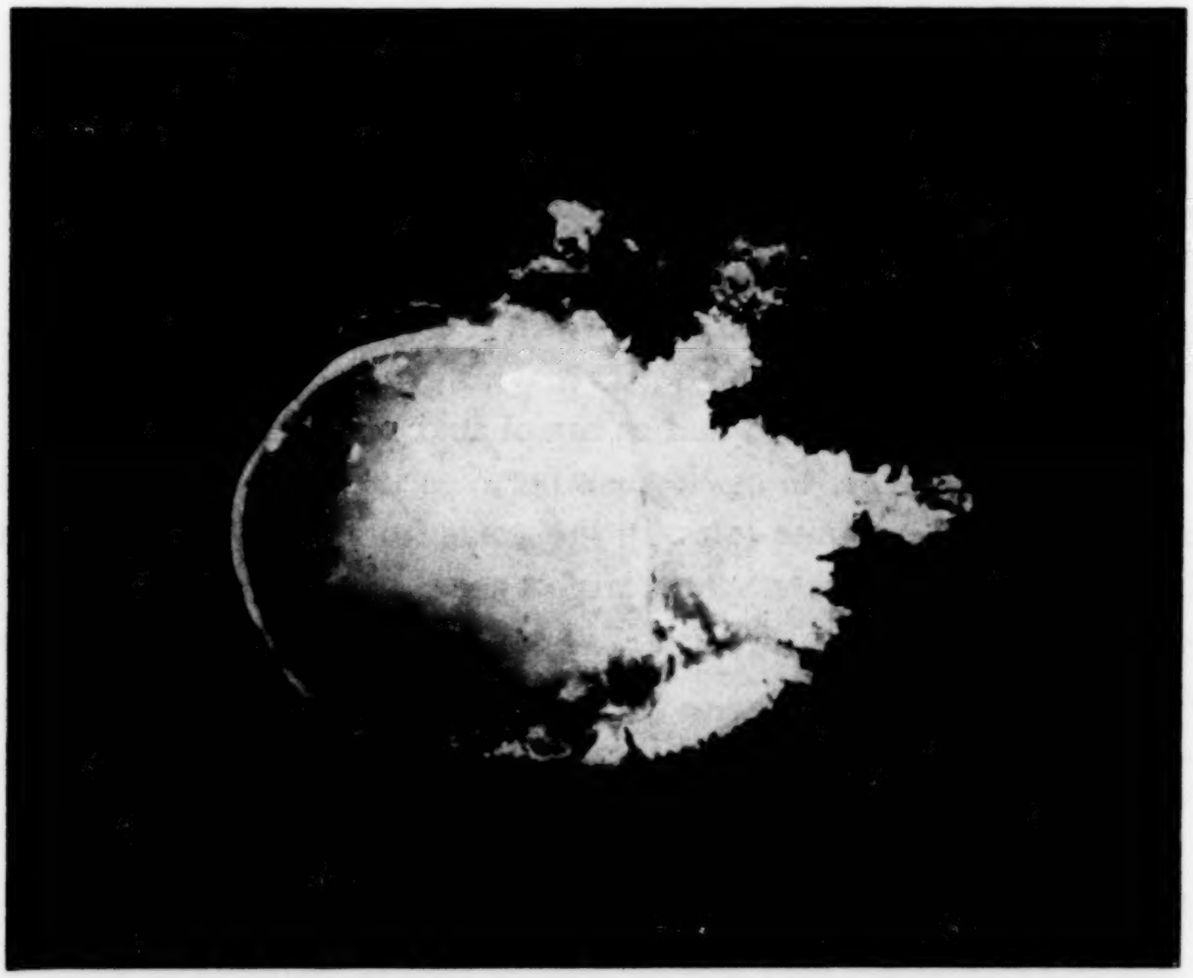

Fig. 6. Sodium isovalerate crystals from diffusion of isovaleric acid from water.

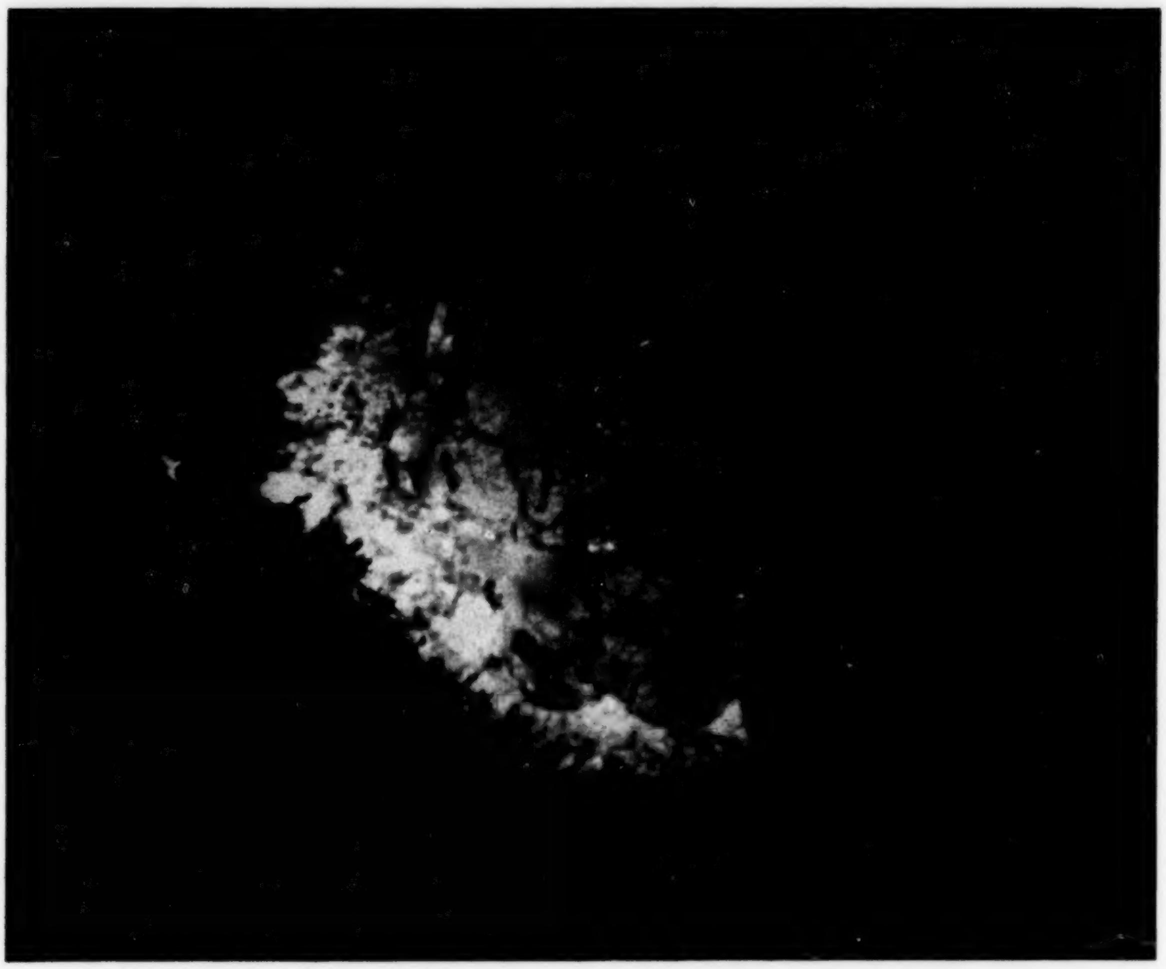

Fig. 7. Product of diffusion of a mixture of formic, propionic and isovaleric acids from water. Note resemblance to Fig. 6.

crystals and the sodium hydroxide pellets dissolved in the water that was formed during the reaction in a very short time. As the volume and concentrations were decreased, and the time increased, there was a crusty growth on the NaOH pellets but there was no differentiation of the acids. This generally occurs when more than 25 mg of acid are used. Between 1 and 25 mg of acid, and at concentrations between 2.0 and 12.5 %, it is possible to distinguish between the acids by the appearance of crystal aggregates of the sodium salts. In the range from 0.5 to 2 mg only the formic and isobutyric acids produce crystals large enough to be distinguished from the very poor crystals formed by the acetic and isovaleric acids. Below this quantity there was no crystal formation.

SUMMARY

A technique is presented for the characterization of certain volatile organic acids in dilute solution. The method may also be used to prepare crystals of the sodium salts for subsequent optical study.

Reference p. 625.

TABLE I

CRYSTAL FORMATION DURING MICRODIFFUSION OF ACIDS TO SODIUM HYDROXIDE

Acid	*Solvent**	*Volume ml*	*Temperature***	*Time h*	*Result*	*Fig.*
Formic	C	2	23°	2	Fine dendritic crystals	1
	B	2	23°	2	Fine dendritic crystals	2
Acetic	C	2	23°	2	Short, stubby crystals	3
	B	2	23°	2	None***	4
Propionic	C	2	23°	2	None	
	B	2	23°	2	None	
	W	0.2	31°	2	None	
Butyric	W	0.2	31°	2	None	
Isobutyric	W	0.2	31°	2	Very fine "cobwebby" crystals	5
Valeric	W	0.2	31°	2	None	
	W	0.2	31°	4.5	One short crystal *ca.* 1 mm long	
Isovaleric	W	0.2	31°	2	Sturdy, rather grainy crystals	6
Isohexanoic	W	0.2	31°	4.5	Cobwebby crystals (similar to isobutyric) growing in the inner glass wall of the inner well of the cell	
Hexanoic	W	0.2	31°	4.5	Short crystals (similar to acetic) growing on inner wall of the inner well of the cell	
Octanoic	W	0.2		16	None	
Formic + propionic + isovaleric	W	0.2	31°	3.5	Sturdy, rather grainy crystals	7
Formic + propionic	W	0.2	31°	3.5	None	
Valeric + isovaleric	W	0.2	31°	3.5	None	

* Acid concentrations approximately 2%. Solvents: C = cyclohexane, B = benzene, W = water.
** 23° is room temperature.
*** An indefinite wrinkled film forms on the pellets in all cases.

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SPOT DISTRIBUTION AND SIZE IN PAPER CHROMATOGRAPHY*

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The chromatographic technique has undergone such wide application and varied development that extensive reviews of even the current literature are often merely bibliographies covering a short period and convey little information beyond the listing of references. The individual interested in one particular aspect of chromatography is seldom satisfied with a few short sentences in several reviews. We are attempting to expand the treatment of individual branches of paper chromatography by correlating experimental and theoretical treatments. The results are partly review and partly original in character. It is hoped that the reader may inform himself as to previous work on one particular facet of the field and simultaneously obtain some direction, based on theory, in his inquiry. The purpose of theory, after all, is to direct research and initiate new ideas. This first paper treats the spot-area and spot-length technique in quantitative paper chromatography. Subsequent communications will deal with multi-zoning, multi-spotting, and tailing.

FISHER, PARSONS AND MORRISON[6] found in the paper chromatography of a number of amino acids, and xylose and arabinose that for regular ovoid spots, (1) the maximum length of the developed spot was linearly related to the logarithm of the quantity of material contained in the spot, and (2) the area of the spot, as measured by a planimeter, was a linear function of logarithm spot-content. Since the size of the spot depends on the extent of development, the paper, solvent, and temperature, they suggest that standards of known concentration be run simultaneously on the same sheet of paper with the unknowns. FISHER AND HOLMES[7] claim an accuracy of ± 5 % for samples of 1 to 2 μg NH_2-nitrogen with the second method, the convenience of the first method being opposed by lessened accuracy which is theoretically expected. FISHER, PARSONS AND HOLMES[8] developed four samples on a single sheet. Two spots were for a standard and two for the unknown. The ratio of the concentrations of the material in the standards was made equal to the ratio of the concentrations of the material in the unknowns by dilution. The formula they present relating spot-size and spot-content depends upon a logarithmic relation between the two. In the case where the areas are difficult to determine because of diffuse boundaries, the authors[6] suggest copying the developed chromatograms

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with a reflex copier. Overexposure gives a clearer definition of the spot on the copy.

BLOCK[3] applied this method, among others, to the analysis of amino acids, cautioning that the spots must be clearly separated and have distinct boundaries. REID AND LEDERER[20] used a modification of the method in the ascending chromatography of the ammonium salts of the volatile C_2–C_7 fatty acids. Two samples of known concentration were run with 6 or 7 unknowns on the same sheet. The knowns, with concentrations at the extremes of the unknowns, gave the linear regression line (spot-area *vs.* log spot-content) from which the content of the unknowns could be determined. Four sheets with the same samples were run to compensate for variation in the paper but more particularly for variations in the revelation of the final spots with a color reagent. Use of a planimeter for spot-areas gave an error of as high as 40%. They preferred to determine areas by tracing the spot onto ruled graph paper for which they report an error of 2 to 5%. ÅKERFELDT[1] found the linear relationship of spot-area to log spot-content to be true in the two-dimensional paper chromatography of amino acids if the initial zone before development is formed by one application. If, however, the zone is formed drop by drop, with not more than 4 drops of 5 μl each (spot-content 10–30 μg) and the paper dried after each drop, then there is a linear relationship between area and spot-content on development in the first direction. The logarithmic relation is regained on development in the second direction. It may be that the first application overloaded the chromatogram so that the concentration fell in a non-linear region of the adsorption isotherm. Development in the first direction would correct this situation since considerable dilution would result. Final spots were cut out and weighed. Errors ranged from 0 to ± 10% depending on the number of determinations.

BLUMER[4] reports a linear relation between spot-area and spot-content for the porphyrins in the range 0 to 0.5 γ with ± 10% error.

In inorganic chromatography BEERSTECHER[2] reported a linear relationship between spot-area and spot-content for potassium ion developed by an ascending technique. Addition of known amounts of potassium salts to the sample makes it possible to use the sample as both a standard and unknown simultaneously. SEILER, SORKIN AND ERLENMEYER[21] found a linear relation for sodium, potassium, calcium, and magnesium chromatographed as their acetates using an ascending technique and revealed with violuric acid. Again, standards of known concentration of these ions were chromatographed along with the unknowns. Difficulties were encountered with sodium and calcium ions in the paper and the separation of sodium and potassium when the ratio of the former to the latter exceeded 5:1. Variation of spot-area with height of ascent was also studied and a plot of the ratio of these two quantities *versus* the quantity of material was nearly linear but results were not very satisfactory since the error was 10 to 15%. Evaluation of (spot-area)$^{\frac{1}{2}}$/(height of ascent) for a given amount of solute showed even greater deviation from an expected constant value. The visual comparison method used by WEISS, FALLAB AND ERLENMEYER[23] for the semiquantitative determination of uranium, iron, and copper also implies a linear dependence. SEILER, SORKIN AND ERLENMEYER[22] applied the spot-area technique to the analysis of sodium, potassium, calcium, and magnesium in serum and urine. It is interesting to note that samples were applied by successive applications of material to the paper with drying between additions. This is similar to the technique of ÅKERFELDT who, under these circumstances, also obtained a linear relation.

LADENBAUER[14] describes a semiquantitative procedure for germanium based on spot-areas.

MODREANU[18] carefully investigated the spot-area technique applied to the propionates of barium and strontium. This investigation is superior in that samples of a mixture of the two ions were applied in one application of a constant volume. For an ascending technique, the relation between spot-area and spot-content was definitely not linear. Unfortunately, other relationships were not tested. We feel that where standards are employed along with the unknowns and the range of concentration is not large, a linear relation between spot-area and spot-content is very likely within experimental error and deviations from linearity are not enough to be detected.

Possibly in an attempt to overcome the difficulty in determining the border of a diffuse spot, FOWLER[10] chromatographed sucrose by an ascending technique on 5 mm wide papers, the solute extending across the paper. The length of the spot was more easily determined. He found that the linear relationship between spot-length and log spot-content failed above 100 to 250 μg sucrose but that a linear relation between log spot-content and log spot-length was valid in the range tested (4 to 450 μg of solute). He found that the spot-length decreased with the temperature, increased with the development time, and increased with the equilibration time before developing the chromatogram. MORI[19] cut parallel slits 2 to 5 mm apart and 30 to 100 mm long at the expected position of the developed spot on the filter paper. The paper outside of this "quantitative bridge" was blocked off with paraffin so the developer was forced to pass through this bridge. Using 0.5 to 10 γ of auramine he also found a linear relationship between log spot-length and log spot-content. MIYAKI, SATAKE AND HAYASHI[16] used 7 mm × 40 cm strips of paper applying 0.1 ml of solutions of several different types of solutes in spots 7 × 7 mm and developed these with five different types of solvent systems. Numerous reagents were used for revelation. Two types of spots were obtained, one due to partition chromatography which is widely accepted as involving a linear retention isotherm and one due to adsorption chromatography which implies a non-linear isotherm. Surprisingly enough they report the following relation for both types:

$$\log\,[(\text{final spot-size}) - (\text{initial spot-size})] = K_1 \log\,(\text{spot-content}) + K_2 \qquad (1)$$

They also plotted spot-size* *versus* the logarithm of the dilution, taking the most concentrated solution as unity. The dilution is, of course, the reciprocal of the concentration. A linear relation was obtained. In a later publication, however, MIYAKI, MOMIYAMA AND HAYASHI[17] reported

$$(\text{spot-length}) = K_1 \log\,[\log\,(\text{dilution of sample})] + K_2 \qquad (2)$$

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* We interpret spot-size as spot-length since the initial spot most certainly extended across the strip under the conditions stated.

for thiamine. In the range 50–1000 γ, FISHER's relation and the two relations of these authors were satisfactory. In the range 8–4000 γ, FISHER's formula failed and eqn. (1) was more satisfactory. They advise that quantitative methods be based on investigations with solutions of known concentration to establish the relation appropriate to the solute being investigated.

FLOOD[9] dipped the edge of blotting paper impregnated with aluminum hydroxide into solutions of inorganic cations allowing the solution to penetrate 1 to 3 cm into the paper. This edge was then dipped in water until 10 cm or more of paper was wetted. The final zone extended upward from the point of application a distance h which was a linear function of the cation concentration. The minimum error was ± 2%. Cation retention seemed to be by ion exchange. Since the kinetics of the process seem radically different from those considered here it is not surprising that the results differ from the empirical and theoretical observations we treat.

BRIMLEY[5], assuming that a spot spreads by diffusion only, borrowed equations from heat-flow theory and theoretically derived the linear relationship between spot-area and log spot-content and the expected error observed by FISHER *et al.*[6]. He stressed, on theoretical grounds, the importance of small, uniform initial spots and prolonged development as improving the accuracy of the method. FISHER *et al.*[8] objected to BRIMLEY's work on the grounds that a diffusion treatment ignored the partition theory of MARTIN AND SYNGE[15] and that it led one to expect circular spots where instead observed spots are elliptical. This objection is valid since there is a spreading in the flow direction due to the kinetics of partitioning in addition to spreading of diffusion. In general, the final spots have a greater extension in the flow direction than perpendicular to it. The situation can be clarified by considering diffusion in a more general way as was done by KAUMAN AND BAK[12] and by GIDDINGS[11]. In a manner similar to ours, KAUMAN AND BAK assume that a molecule participating in an exchange between the mobile and immobile phase interacts with the mobile fluid in a manner which may be described by a diffusion which is in addition to ordinary diffusion in the mobile phase. Assuming the initial distribution to be a δ-function, all of the material to be in the immobile phase, no secondary transformations and no diffusion in the fixed phase, they solve a set of differential equations for the ratio of the spot-length to spot-width in terms of effective diffusion coefficients in these directions and show that the final spot is elliptical. This ratio may also be expressed in terms of rates of adsorption and desorption, the velocity of the developer and a simple diffusion coefficient. Here we also consider the partitioning process to be described by diffusion and applying this model to quantitative paper chromatography.

First consider spreading of a spot or band in the flow direction on development. The three sources of this spreading are: (1) ordinary diffusion, (2) "eddy" diffusion caused by the random path of molecular flow in a porous medium, and (3) the kinetics of partitioning responsible for local non-equilibrium effects[11]. Each of these sources is responsible for a spreading that obeys the law of diffusion, and each can be assigned an effective diffusion coefficient[11,12]. The total spreading is determined by a net diffusion coefficient, D_z, which is the sum of the three individual coefficients. The z-direction is taken as the direction of developer flow or longitudinal flow. A similar argument applies to spreading perpendicularly or laterally to the flow, the y-direction, except that there is no contribution from the third source. The net diffusion coefficient, D_y, is the sum of coefficients for ordinary molecular diffusion and "eddy" diffusion. The latter is probably numerically different in the two directions.

The spot has a greater extension in the z-direction than in the y-direction because D_z, a trinomial sum, is ordinarily greater than D_y, a binomial sum. The empirical linear relation between spot-area and log spot-content can be readily demonstrated by quantitative consideration of this asymmetric diffusion.

In the simplest case the initial spot as applied to the paper is small compared to the developed spot and may be approximated by a point source. The migration of the center of a symmetrical spot is determined by the R_F value while spreading is determined by D_z and D_y. Consider the case where n molecules are applied at the origin. At a time t after development is started the number of molecules in the region z to $z + \mathrm{d}z$ is

$$n_z \mathrm{d}z = \frac{n}{\sqrt{4\pi D_z t}} \exp\left[-(z - \bar{z})^2/4D_z t\right] \mathrm{d}z \qquad (3)$$

where $\bar{z}$ is the displacement of the center of the developed spot from the point of application. The analogous equation for the number of molecules in the lateral direction lying between y and $y + \mathrm{d}y$ is

$$n_y \mathrm{d}y = \frac{n}{\sqrt{4\pi D_y t}} \exp\left[-(y - \bar{y})^2/4D_y t\right] \mathrm{d}y \qquad (4)$$

where $\bar{y}$ is the lateral displacement of the center of the developed spot from the point of application; it is normally zero. The number of molecules per unit area, or number density, is $n_z n_y/n$ or

$$d = \frac{n}{4\pi \bar{D} t} \exp\left[-\frac{(z - \bar{z})^2}{4D_z t} - \frac{(y - \bar{y})^2}{4D_y t}\right] \qquad (5)$$

where $\bar{D}$ is $\sqrt{D_z D_y}$.

When the number density falls below a certain value, say d_0, the dilution is such that the solute cannot be detected. This limit depends upon the sensitivity of the method of detection whether it be visual, radioactive counting, photoelectric densitometry, etc. Once d_0 is determined than eqn. (5), with d replaced by d_0, defines the observable boundary of the spot in the y,z-plane. In logarithmic form

$$\ln d_0 = \ln n - \ln 4\pi \bar{D} t - \frac{(z - \bar{z})^2}{4D_z t} - \frac{(y - \bar{y})^2}{4D_y t} \qquad (6)$$

which rearranges to

$$\frac{(z - \bar{z})^2}{4D_z t \ln (n/4\pi d_0 \bar{D} t)} + \frac{(y - \bar{y})^2}{4D_y t \ln (n/4\pi d_0 \bar{D} t)} = 1 \qquad (7)$$

This is the equation of an ellipse with center at $\bar{z}$, $\bar{y}$. The semi-major axis, a, in the

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z-direction, half the length of the spot, and the semi-minor axis, b, in the y-direction, half the width of the spot, are given by

$$a = [4D_z t \ln (n/4\pi d_0 \overline{D}t)]^{\frac{1}{2}} \tag{8a}$$

$$b = [4D_y t \ln (n/4\pi d_0 \overline{D}t)]^{\frac{1}{2}} \tag{8b}$$

$a > b$ since $D_z > D_y$. The area, A, of the ellipse is πab or

$$A = 4\pi \overline{D}t \ln (n/4\pi d_0 \overline{D}t) \tag{9}$$

One concludes that even with asymmetric diffusion ($D_z \neq D_y$), the area of an ideal spot is linear in the logarithm of sample size.

Some other significant conclusions from this treatment are:

1. The elliptical shape of the spots is in accord with the comments of FISHER *et al.* A slight amount of tailing in many cases is responsible for ovoid or egg-shaped spots rather than ellipses.

2. Owing to the slowly varying logarithmic term, the spot area initially increases nearly linearly with time. If the velocity of developer flow were constant, the distance from the point of application to the final spot would be proportional to time and spot-area would be proportional to this distance for a given amount of material. This velocity is not generally constant and we would not expect expressions as simple as those tested by SEILER, SORKIN AND ERLENMEYER[21] to be valid. Furthermore the values of D_z and D_y may vary as a result of velocity changes. In this case average values must be used for these quantities. At time $t = n/4\pi d_0 \overline{D}$, the spot has become so dilute that it is undetectable and $A = 0$. This time is longer than usually encountered experimentally.

3. The length of the spot, equal to $2b$, is expected to increase in proportion to the square-root of the logarithm of spot content. This disagrees with statements in the literature. It has been remarked that spot-area is more accurate than spot-length[6]. Part of this inaccuracy may be due to an erroneous assumption for the relationship between length and spot-content. In addition, percentage wise, lengths are more sensitive to errors of measurement than areas.

4. It can be shown that any initial spot for which the number density is originally an asymmetric Gaussian distribution,

$$d = \frac{n}{2\pi\sigma_y\sigma_z} \exp\left[-\frac{(z-\bar{z})^2}{2\sigma_z^2} - \frac{(y-\bar{y})^2}{2\sigma_y^2}\right] \tag{10}$$

appears then and at any later time as an ellipse regardless of the original values of the standard deviations. The initial spot may even be an ellipse with its major axis perpendicular to the direction of developer flow. In any event, after a time t of development the semi-axes are given by

$$a = \left[(2\sigma_z^2 + 4D_z t) \ln \frac{n}{2\pi d (\sigma_z + \sqrt{2D_z t}) (\sigma_y + \sqrt{2D_y t})}\right]^{\frac{1}{2}} \tag{11a}$$

$$b = \left[(2\sigma_y^2 + 4D_y t) \ln \frac{n}{2\pi d (\sigma_z + \sqrt{2D_z t}) (\sigma_y + \sqrt{2D_y t})}\right]^{\frac{1}{2}} \tag{11b}$$

The spot-area is still linear in log spot-content. These considerations are mentioned to account for the finite size of the initial spot before development. While an asymmetric Gaussian distribution cannot be assumed as the original condition in all cases, it is probably as good as other assumed forms such as the circle of uniform concentration of BRIMLEY[5] or the δ-function of KAUMAN AND BAK[12]. Owing to the uncertainty of the concentration distribution of the original spot it is desirable to keep it small compared to the developed final spot as BRIMLEY has pointed out. Otherwise, owing to this uncertain concentration in the initial spot, a definite relationship between spot-area and spot-content cannot be stated. Techniques such as very rapid development where diffusion does not significantly alter the shape of the original spot are strongly dependent on the method of application of the sample and are not considered here.

5. If the solute is applied as a thin line across the strip, only diffusion in the z-direction need be considered. In this case the number density becomes

$$d = \frac{n}{w\sqrt{4\pi D_z t}} \exp\left[-(z-\bar{z})^2/4D_z t\right] \tag{12}$$

where w is the width of the strip. When d_0 is substituted for d and eqn. (12) rearranged, the length of the zone becomes

$$2(z-\bar{z}) = \left[16D_z t \ln\left(n/d_0 w\sqrt{4\pi D_z t}\right)\right]^{\frac{1}{2}} \tag{13}$$

or proportional to the square-root of log spot-content.

Unfortunately FOWLER presents no data and MORI's method of channeling the solute into a narrow strip after some development has occurred varies enough from the model presented here as to be suspect. The spot, as it enters the bridge, is probably significantly large and wider than the bridge, although MORI does not comment on this.

The length of the zone is expected to increase, except for the slowly varying logarithmic term, with the square-root of time. FOWLER states that the length increases with time but does not specify the relationship.

The diffusion coefficient, D_z, is not itself simple. It will depend upon the kinetics of the phase transfer[11], ordinary diffusion, and eddy diffusion and how these, in turn, are affected by temperature, developer flow rate, characteristics of the paper, etc. This justifies the observation of FISHER, PARSONS AND MORRISON that each sample is best run simultaneously with a standard so that the parameters affecting the spot-size are the same in the two cases.

Ultimately the choice of relations between spot-area and spot-content rests upon the conditions of the individual experiment. The decision as to whether the theoretically more accurate but more complex relations are warranted in view of accompanying experimental errors must be made by the researcher concerned.

SUMMARY

Previous experimental and theoretical work on quantitative paper partition chromatography by spot-area and spot-length is reviewed. An asymmetric diffusion model for chromatography, where diffusion in the direction of developer flow and perpendicular to it are unequal by virtue of the kinetics of partition, is applied to derive the relationships that spot-area is directly proportional to log spot-content, and spot-length is directly proportional to square-root of log spot-content. Relations for the increase of spot-area and spot-length with time are given.

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REDUCTION CHROMATOGRAPHY OF CATIONS

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INTRODUCTION

When searching for phenomena that could give rise to chromatographic separations our attention was drawn to the possibility of separating metals on the basis of the differences in the values of their normal potentials.

We anticipated that chromatographic separation could occur on passing a mixture of cations through a chromatographic column packed with powdered metal, if the normal potential of this metal were lower than that of the cations passing through. The ions would be successively displaced from the solution in the order of decreasing normal potential.

In the chromatographic method based upon this principle of displacement of metals from the solutions of their ions, the quality of the separation does not depend directly on the reduction potentials of the metals, but on the reduction equilibrium constants. These values can be easily calculated.

For example for the equilibrium state of the reaction:

$$\mathrm{Fe}^{\circ} + \mathrm{Pb}^{++} \rightleftharpoons \mathrm{Fe}^{++} + \mathrm{Pb}^{\circ}$$

the following equation holds:

$$E_{\circ\ \mathrm{Fe}^{\circ}/\mathrm{Fe}^{++}} + E_{\circ\ \mathrm{Pb}^{++}/\mathrm{Pb}^{\circ}} = \frac{RT}{nF} \ln K$$

Insertion of the corresponding values gives:

$$0.440 - 0.126 = \frac{8.316 \times 298.1 \times 2.303}{2 \times 96494} \log K$$

Hence:

$$K = \frac{a_{\mathrm{Fe}^{++}}}{a_{\mathrm{Pb}^{++}}} = 4.17 \cdot 10^{10}$$

The equilibrium constants of the displacement reactions of the ions Cd^{++}, Co^{++}, Ni^{++}, Sn^{++}, Cu^{++} and Ag^{+} with iron, calculated as above, are the following:

$$\frac{\mathrm{Fe}^{++}}{\mathrm{Cd}^{++}} = 19.15$$

$$\frac{\mathrm{Fe}^{++}}{\mathrm{Co}^{++}} = 3.24 \cdot 10^{5}$$

$$\frac{\mathrm{Fe}^{++}}{\mathrm{Ni}^{++}} = 7.94 \cdot 10^{6}$$

$$\frac{\mathrm{Fe}^{++}}{\mathrm{Sn}^{++}} = 1.41 \cdot 10^{10}$$

$$\frac{\mathrm{Fe}^{++}}{\mathrm{Cu}^{++}} = 3.16 \cdot 10^{26}$$

$$\frac{\mathrm{Fe}^{++}}{2\ \mathrm{Ag}^{+}} = 6.31 \cdot 10^{41}$$

These values show that chromatographic separations accomplished with this method will be very extensive.

EXPERIMENTAL PART

General

A. Chromatographic columns

Chromatographic columns 600 mm long and 10 mm in diameter fitted with stopcocks at the bottom were used.

B. Chromatographic beds

The columns were packed with powdered metals. In our investigations beds of powdered zinc, aluminium and iron were applied.

Beds consisting of zinc and aluminium generally proved to be useless because of the evolution of hydrogen. This occurred not only when separating mixtures of cations from acid solution but also from basic or even weakly basic solutions. It was the main reason why we used beds of powdered iron in our investigations.

We used two different types of powdered iron:

1. Electrolytic iron powder (Swedish), containing 0.09% C, 0.012% Si, 0.022% Mn, 0.0058% S and 0.018% P; the particles were of irregular shape, the size ranging from 0.2–0.4 mm.

2. Technical iron powder produced by "Toxa" (Polish), consisting of spherical grains with diameters ranging from 0.315–0.50 mm.

The size of the grains chosen was such that good physical properties of the bed were obtained. Its hydraulic resistance must be sufficiently small and must not undergo any changes when metals are deposited on the bed. The columns were packed wet (in suspension) without suction being applied. Uniform packing of the beds was achieved by repeatedly tapping the columns with a wooden stick.

C. Initial solution

Cations were separated from neutral, ammonia and cyanide solutions. The concentration of the cations was 0.01 moles per litre. The concentrations of ammonia and cyanide were as high as necessary for formation of the corresponding complexes.

D. Flow rate

The flow rate was chosen in accordance with the method of separation. It was quite low when the aim was to separate the cations in the form of separate layers, but higher when the frontal method was applied. In experiments where separate chromatographic layers were obtained in the column, the sequence of the deposited cations and the lengths of the layers were determined by visual observation. In the case of frontal chromatography alternate portions of effluent were analysed qualitatively in order to determine the sequence and the extent of separation of the ions. Polarography was applied to examine more closely the shape of the front as regards the deposited ions; the changes in the amount of iron passing into solution were determined colorimetrically.

Experimental details

A. Separations by means of classical chromatographic methods

These were carried out on beds of zinc, aluminium and iron. The results of the experiments proved unsatisfactory. Clearly distinct layers were obtained only for silver and copper cations in neutral and ammoniacal solutions. In all cases a distinct silvery grey layer of deposited silver was formed at the top of the bed immediately followed by a red layer of deposited copper. An interesting feature of the influence of the presence of silver on the formation of the copper layer was observed. When silver ions are not present in the solution, the copper ions are deposited in the column as wide, poorly separated layers. The width of the layers depends on the concentration of the cupric ions and on the flow rate. When a mixture of silver and copper cations is passed through the column, the copper layer is much narrower and well shaped, the width being only slightly dependent on the flow rate. We suppose this is due to the creation of a copper–silver pile working on the column.

Other cations give layers that are wider and more difficult to determine by means of visual observation. For example lead cations are deposited after silver and copper in the form of a dark grey, wide, poorly shaped layer.

B. Experiments in which frontal analysis was used

Very interesting results were obtained when the separation of cations in the effluent from the column was investigated. Frontal analysis was applied to separate the nitrates of silver, copper, lead, nickel and cadmium on a chromatographic bed of iron powder ("Toxa"). The experimental conditions were the following: diameter of the column: 9.9 mm; height of the bed: 350 mm; empty space: 12 ml; concentration of cations in the influent: 0.01 *M* with respect to each cation; flow rate: approx. 0.2 ml/min. The content of the effluent was determined by qualitative analytical methods.

The results of this experiment are given in Table I.

TABLE I

Cation	*Presence of cations in the effluent (ml)*																	
	2	*4*	*6*	*8*	*10*	*12*	*14*	*16*	*18*	*20*	*22*	*24*	*26*	*28*	*30*	*32*	*34*	*36*
Fe^{++}	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+
Cd^{++}	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+
Ni^{++}	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+
Pb^{++}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
Cu^{++} Ag^{+}	not found in the effluent. Two clearly distinct layers at the top of the bed; upper layer: silver; lower layer: copper																	

As can be seen, the sequence of the ions appearing in the effluent is in the order of the increasing normal potentials in neutral solution.

An analogous experiment was carried out using a bed of powdered electrolytic iron under the following conditions: column diameter: 9.9 mm; height of the bed: 140 mm; empty space: 4.8 ml; concentration of the influent solution: 0.01 *M* with respect to each cation.

Fractions of 1 ml were collected and analysed. The following sequence of cations in the effluent was established: cadmium–nickel, lead. Silver and copper were completely adsorbed on the column, where they formed two clearly distinct layers (copper below silver); their ions were not found in the effluent.

In order to determine the shape of the front of the effluent in the method we have proposed, an experiment was carried out in which lead and cadmium were separated from each other. The conditions were as follows: column diameter: 9.9 mm; height of bed of powdered electrolytic iron: 202 mm; empty space: 6.8 ml; initial concentration of solution: 0.01 *M* with respect to each cation; flow rate: approx. 0.07 ml/min. The concentration of cations in the effluent was determined polarographically. At the same time the shape of the front of the displaced iron was investigated colorimetrically with ammonium rhodanide, using Hilger's "Spekker" photo-colorimeter. The results are shown in Fig. 1, where the concentrations of the cations investigated are plotted against the volume of the effluent.

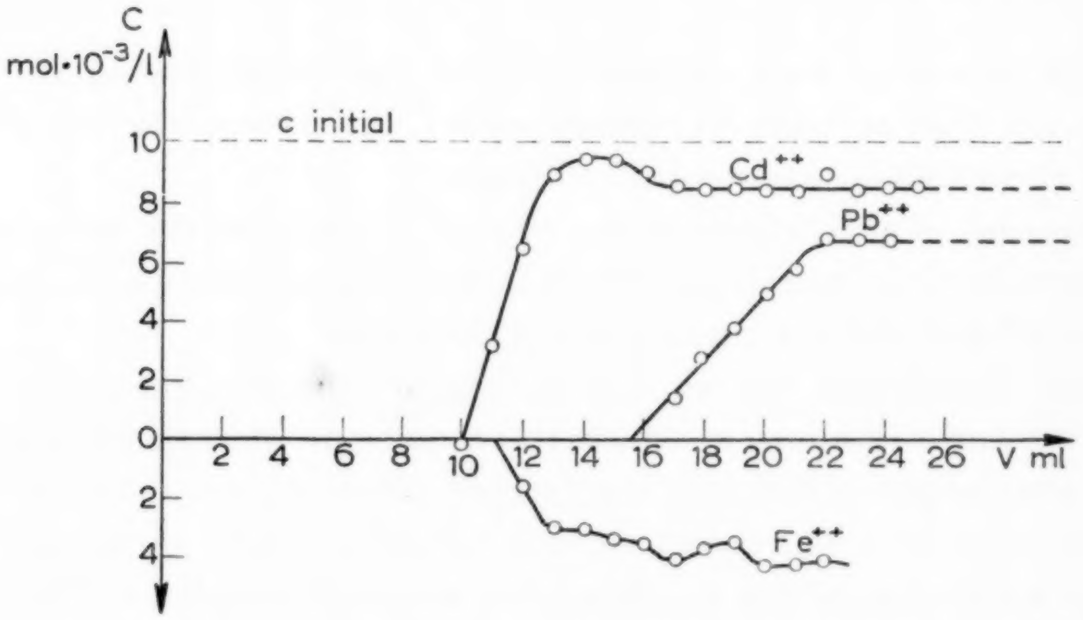

Fig. 1. Changes of the ion concentrations and displaced iron in the effluent from the chromatographic column.

In this figure it can be seen that the fronts of cadmium and lead have a very regular shape, whereas that of iron is irregular.

It is interesting that the concentrations of cadmium and lead in the effluent reach a constant value below that of their initial value, which is shown in the figure by a horizontal dashed line.

The separation of different cations in the front of the effluent, using an ammoniacal solution was also investigated. The conditions were as follows: column diameter: 9.9 mm; bed of powdered "Toxa" iron; height of the bed: 137 mm; empty space: 7.0 ml; the concentration of the cations $Ag(NH_3)_2^+$, $Cu(NH_3)_4^{++}$, $Ni(NH_3)_4^{++}$ and $Cd(NH_3)_4^{++}$ was 0.01 *M* with respect to each complex cation. The concentration of the ammonia was as high as was necessary to obtain the required complexes. The flow rate was approx. 0.1 ml/min. The presence of the examined cations was established by qualitative analytical methods. The results of this experiment are given in Table II.

TABLE II

Cation	*Presence of cations in the effluent (ml)*																	
	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Fe^{++}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cd^{++}	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
Ni^{++}	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
Cu^{++}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+
Ag^{+}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Nickel is not separated from cadmium, which can be attributed to the fact that the bed was not high enough. As regards copper, this appeared in the effluent sooner than in the experiments with neutral solutions.

The sequence of the cations in the effluent is therefore the same as that of the normal potentials of the metals concerned in ammoniacal solution. It must be pointed out that the effluent did not contain any ferrous ions.

A similar experiment was carried to separate the cyanide complexes of silver, copper, nickel and cadmium, in which the conditions were as follows: column diameter: 9.9 mm; height of bed: 123 mm; empty space: 4.5 ml; initial concentration of the solution: 0.01 M with respect to each complex cation. Potassium cyanide was added in an amount sufficient to obtain the required complexes. The flow rate was about 0.1 ml/min.

Table III shows the results of this experiment.

TABLE III

Cation	*Presence of cations in the effluent (ml)*																	
	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
Fe^{++}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cd^{++}	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+
Ni^{++}	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cu^{++}	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ag^{+}*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* The first traces of Ag^{+} appeared in the 54th ml of effluent.

The data obtained show that copper appears in the effluent before other cations, in spite of the fact that its normal potential in cyanide solution is only — 0.43, whereas for cadmium the value of the normal potential is — 1.03. The main cause of this phenomenon seems to be the very small value of the instability constant of the copper complex ($K = 5 \cdot 10^{-28}$) compared with that of other cations. This experiment was prolonged until all the separated cations appeared in the effluent. The first traces of silver ions appeared in the 54th ml of effluent. No ferrous ions are observed in the effluent.

SUMMARY

1. A new method of separating cations—reduction chromatography—is proposed.

2. It was shown that chromatographic separation can be based on the differences of the normal potentials of metals, since these differences give rise to mutual displacement.

3. Frontal analysis of several cations in neutral, ammoniacal and cyanide solutions on a bed of powdered iron has been carried out.

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ELECTROLYTIC CHROMATOGRAPHY

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It is difficult to find common features among the enormous variety of contemporary chromatographic methods, on the basis of which the methods could be grouped into one class. There is no doubt that one of the phenomena common to all chromatographic processes is the presence of two phases that are moving in relation to each other. One of these phases always contains the mixture being examined and this undergoes separation along the phase boundary (either diffusing into the other phase or not). A further characteristic of chromatography is that the separation is accomplished by material counter-current.

As regards the causes of chromatographic separation, these can be various and it is not advisable to consider them as common features of chromatographic methods.

In the chromatographic methods of separating ions that have been carried out up to the present, several principles are applied. Some of these methods are based upon the differences of solubility of poorly soluble compounds that can arise on the surface of one of the moving phases, while others depend upon the differences between the exchange coefficients of ions on phase boundaries, or on the differences of partition coefficients between two solvents.

These considerations lead to the conclusion that it should be possible to obtain chromatographic separation of ions by making use of other properties.

In our present work we were interested in examining the possibilities of carrying out chromatographic separations of ions by making use of the differences in their reduction potentials. A method based on this principle does not seem to have been considered up till now.

If conditions could be created in which the moving phase, consisting of a mixture of cations, flows past motionless electrodes so that it meets a gradually increasing potential difference, the ions should be successively separated when their reduction potentials are exceeded. Such an arrangement of electrodes supplying a gradually increasing potential difference can be realized in a manner shown in Figs. 1a and 1b.

In the scheme shown in Fig. 1a the potential difference from the direct current source (2) is applied to an electrode (1), the electric resistance of which is significant. The electrode is placed in a chromatographic column (4), which is first filled with a liquid that is immiscible with the solution being examined. This liquid is a non-conductor and its specific density is higher than that of the solution under investigation. Mixed solutions of cations are placed on the top of the inert liquid and passed down with a velocity that can be controlled.

In such a system the potential of the electrode (1) is variable because the potential difference between the top of the electrode and the point where it intersects the moving boundary of the two liquids (point 6) steadily increases as the liquid passes down the column. These conditions provide a means of separating ions chromatographically according to the frontal method. A suitable amount of solution is continuously applied to the top of the column; owing to the electric conductivity of the solution a circuit is established through the solution between the top of the electrode and point (6) on the three phase boundary.

At any moment when the potential difference between these two points exceeds the reduction potential of any of the cations in the solution, an electrolytic process begins. The anode is then the top end of the electrode and the cathode is the point on the electrode where the three phases meet.

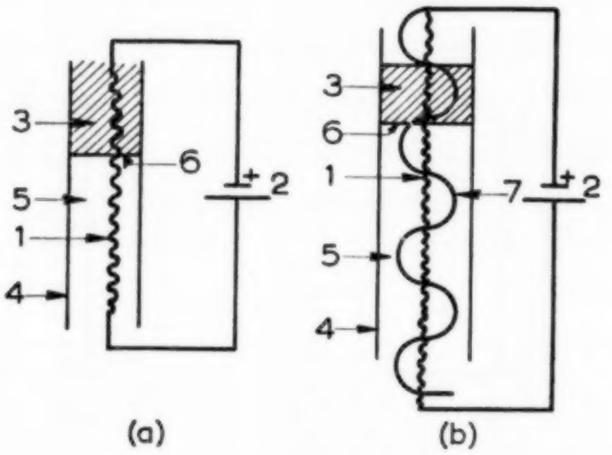

Fig. 1. Schemes of one and two-electrode arrangements for electrolytic chromatography. 1 = electrode of variable potential; 2 = direct current source; 3 = solution being examined; 4 = chromatographic column; 5 = supporting liquid; 6 = liquid–liquid interface; 7 = electrode of constant potential.

In such conditions the cations with the lowest reduction potentials will be reduced first, while those with higher potentials will only be reduced after the solution has passed further down the column. It is evident that, as in the case of other frontal chromatography methods, complete separation of a mixture of substances cannot be achieved in this way. A complete separation of cations is possible with the system shown in Fig. 1b.

Besides the electrode (1) of variable potential another electrode (7) with a very small electrical resistance is introduced. The potential of this electrode is equal to that at the top of electrode (1) to which it is connected.

While the thin layer of solution (3) is being carried down with the non-conducting liquid (5), the potential difference between the electrode of constant potential and that of variable potential will increase continuously. After the reduction potential of the first cation has been exceeded, an electrolytic process will start. Dependent on the potential applied, either the electrode of constant potential (7) or that of variable potential (1) will be the cathode.

If the flow rate is not too high, the ions should be reduced in separate layers in the order of increasing reduction potentials.

GENERAL EXPERIMENTAL PART

Electrode of variable potential

The electrical resistance of this electrode, which is connected to the current source, should be such that cooling by the ambient liquid is sufficient to prevent a perceptible increase of temperature. On the other hand, the electric current flowing through the electrode must be high enough to provide the required rate of electrolysis resulting from the ramification of the current, one part flowing through the solution. This ramification is shown schematically in Fig. 2.

The arrow i_0 represents the current in the circuit before the decomposition potential of any salt in the solution has been exceeded. i_1 and i_2 indicate the ramification of the current between electrode and solution after the decomposition potentials of some of the salts have been surpassed, *i.e.* after the electrolytic processes have begun. The rate of electrolysis depends on the i_0 value and the ratio between the resistance of the electrode and that of the solution.

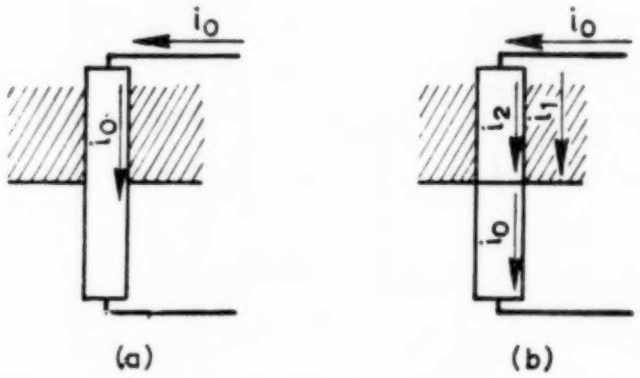

Fig. 2. Flow of current in electrolytic chromatography. (a) before the decomposition potential of an ion has been exceeded. (b) after the decomposition potential of an ion has been exceeded. i_0 = initial current; i_1 = current flowing through the solution; i_2 = current flowing through the electrode.

The electrode of variable potential should be made of a material that does not permit the creation of local piles on its surface when it is in contact with an electrolyte solution and of which no ions pass into solution during the electrolytic process. It is advisable that the electrode should have a significant overvoltage in comparison with hydrogen.

The electrode material should also have good adhesive properties for the metals deposited on its surface. If an additional electrode of constant potential is used, this should be made of material with a specific resistance as low as possible, and its own ions should not pass into solution.

It must be pointed out here that the system of these two electrodes must not constitute a working pile with the electrolyte solution under investigation.

The electrodes of variable potential that we used initially were made of kanthal and nickel wire of different cross-section or were graphite rods with some clay admixed; the specific resistances of all these electrodes were different. The electrodes of constant potential were made of platinum wire. In the one-electrode system (Fig. 1) both kanthal and nickel electrodes dissolved at the points that actually constitute the anode and thus caused disturbance of the process.

In the system with two electrodes using a platinum electrode as the electrode of constant potential in conjunction with kanthal or nickel electrodes of variable potential, an electric pile was soon created, which also complicated the process. This is why in two-electrode systems we used a graphite rod as electrode of variable potential and a platinum spiral as electrode of constant potential. A spiral electrode has, however, some disadvantages. It is difficult to obtain a uniform disposition of the spiral coils. Furthermore, a thin layer of liquid flowing down is retained on the coils, especially at the points where they come into contact with the column wall. Platinum net as electrode has similar disadvantages. The best solution to the problem seems to be an arrangement in which the electrode of constant potential is a platinum tube, which serves at the same time as the chromatographic column, the solution and the electrode of variable potential being placed inside the tube. Another possible form is a basket constructed of platinum wires arranged parallel to the wall of a glass chromatographic column, as shown in Fig. 3.

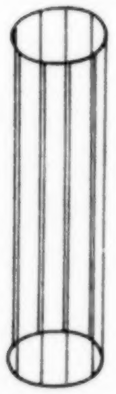

Fig. 3. Platinum electrode as electrode of constant potential.

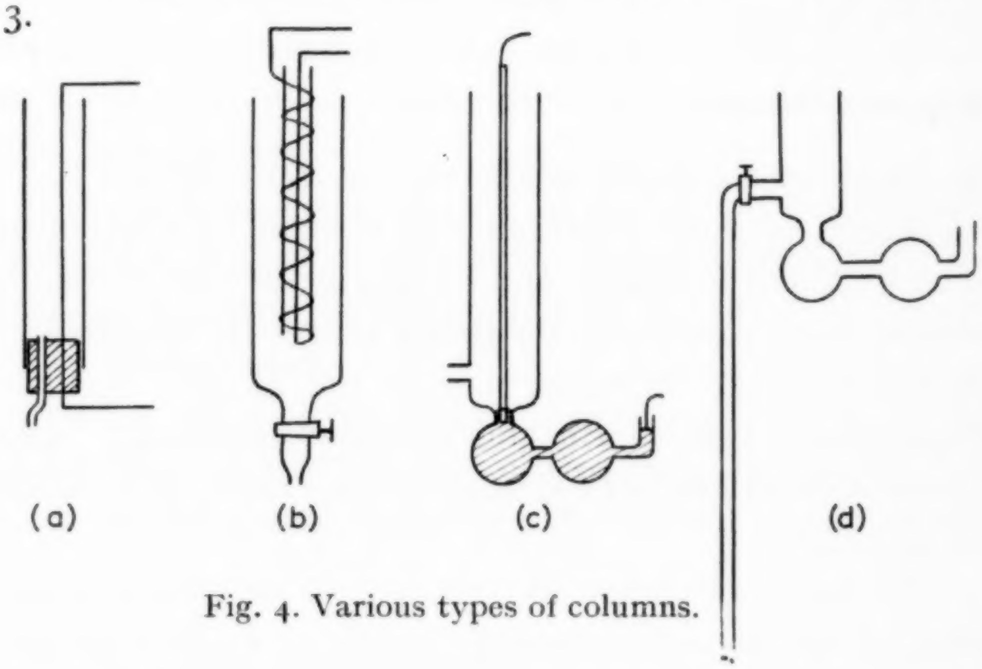

Fig. 4. Various types of columns.

Chromatographic columns

In this work glass tubes were used as chromatographic columns. The ends of the electrodes were led out of the column through the cork in the bottom (Fig. 4a) or through a tiny glass tube introduced into the column (Fig. 4b), or through a mercury seal (Fig. 4c). The last-mentioned method is especially convenient when graphite electrodes are applied. In order to enable the exact regulation of the flow of liquid, a stopcock was placed between the column outlet and the long capillary tube (Fig. 4d); this also minimises the influence of hydrostatic pressure changes on the flow rate.

Composition of electrolyte solutions

In our work we were not very much interested in the influence of the composition of the electrolyte on the quality of the chromatographic separation of the cations, or in the degree of adhesion of the deposited metal to the electrode.

The composition of the electrolyte solutions that we examined were similar to those applied in quantitative electrolytic analysis. Some solutions usually applied in electroplating procedures were also adopted for our purposes.

From the data obtained we attempted to establish what the general composition of the electrolyte solutions should be, apart from specific additions for the individual ions. An electrolyte solution suitable for chromatographic separation according to our method should be a solution:

1. which permits the separation of a great number of different cations;
2. in which the differences of the reduction potentials of cations are large;
3. that does not give rise to the evolution of gaseous products on electrolysis (at least not at the cathode), or destroys the oxidizing action of oxygen evolving at the anode;
4. from which the metals being examined deposit quantitatively as rapidly as possible in the form of strongly adhering layers that do not undergo any further reactions.

It does not seem possible within a short time to find an electrolyte solution that fulfils all the above requirements. In our work we have investigated the possibilities of chromatographic separation by means of electrolysis from sulphuric acid and ammoniacal solutions.

Supporting liquid

The choice of a suitable indifferent liquid to support the test solution in the column is a matter of great importance in electrolytic chromatography. This liquid should be an excellent insulator, and its specific density should be greater than that of the solution being examined. Its ability to wet the electrodes should be less than that of the test solution. Moreover it must not dissolve the salts of the electrolyte under investigation. Chloroform and carbon tetrachloride, which we used in our work, dissolved small amounts of salts in some cases, thus making the quantitative results less accurate.

We have not widely investigated the problem of finding a supporting liquid that fulfils all the above-mentioned conditions. Carbon tetrachloride has been applied in all our work, except for separations from ammoniacal solution, where the solubility of copper salts was very significant. In this case chloroform was used as indifferent supporting liquid.

When a two-electrode system (Fig. 1b) is applied, liquid can be prevented from remaining on the electrodes after the solution has flowed down by coating the electrolyte solution with a layer of another indifferent liquid. This liquid must not dissolve the salts being analysed and the density and wetting angle should be less than those of the electrolyte. When three liquids are applied—indifferent supporting liquid, electrolyte solution and indifferent wash liquid—the following inequalities must be fulfilled: $\rho_l > \rho_e > \rho_w$ for the densities and $\gamma_l > \gamma_e > \gamma_w$ for the wetting angles.

In some of our work ethyl ether was used as a wash liquid.

Assessment of separation

Visual observation is the simplest way to determine the sequence in which the substances being analysed are distributed on the chromatogram. In order to obtain the necessary data for this, electrolytic chromatography of solutions containing only one ionic species was carried out before mixtures were investigated. The layers of metals deposited on the cathode are of different colours or shades, so that their position can be determined visually. In doubtful cases visual observation was confirmed by scraping some of the metal off the electrode and analysing it qualitatively. Qualitative methods were also used to establish the content of the ions in solution after the experiment.

Visual observation, although suitable for confirming the deposition of cations and their separation, is insufficient to evaluate the degree of separation and the composition of mixed layers that occur besides the pure layers of deposited metals.

In our experiments the degree of separation was investigated by means of emission spectrography in an arc of direct current using a middle dispersion spectrograph ISP-22. For the spectral analysis the electrode on which the chromatographic separation had been accomplished was divided into parts, which were then examined separately.

Tests were also carried out to investigate the chromatographic separation process by determining the value of the current i_1 (Fig. 2), that is the part of the total current passing through the solution, from the increase of the current in the circuit caused by the appearance of electrolyte conductivity.

The potential decrease at the resistance R in the circuit containing the electrode of varying potential, is compensated before the electrolysis process begins. Then the value of i_1 due to the change of the potential decrease at R when the current begins to flow through the solution, can be determined.

It appeared, however, that the value of the current i_1 depends not only on the quantity of metal deposited per time unit but also on other factors which we have failed to determine, above all on the gaseous products evolved at the electrodes (hydrogen and oxygen).

Experimental details

Examples of chromatographic separations

Since frontal chromatography does not lead to complete separations, we therefore investigated in general the separation of ions by means of the two-electrode system (Fig. 1b). The following system appeared to be the most suitable of all the systems we examined: a graphite rod as the electrode of variable potential and platinum wire as that of constant potential.

The chromatographic column that we used was 150 mm in length measured from the top to the side outlet tube. The diameter of the column, which was fitted with a mercury seal, was 7 mm. The electrodes were graphite rods 190 mm in length and of different diameters varying between 2 and 3 mm depending on the brittleness of the dried clay product. Their electric resistances were correspondingly 41 Ω, 17 Ω, 9 Ω, 4.5 Ω and 0.5 Ω.

The graphite electrode was connected to the supply battery, the connection at one end being made by a screw and at the other by means of mercury into which the bottom end was plunged. The potential difference between the two ends of the graphite rod was measured with a voltmeter of 0.1 V accuracy.

A constant potential electrode in the form of a closely wound spiral of platinum wire reached from the top of the column to the side tube inlet, so that it surrounded the whole graphite electrode. The outlet tube was joined by means of a rubber tube fitted with a screw clamp, to a glass tube with a rightangle bend (Fig. 4d), the long arm of which formed a capillary tube 500 mm in length. The solution flowed from the column through this capillary into a calibrated cylinder of 10 ml, so that the velocity of the liquid flowing out of the column could be determined.

Table I shows the results of separations of cations of silver, copper, cadmium and zinc. From the data obtained it can be seen that under the conditions applied all these four cations were separated. The conditions of the separations listed above were selected after a great number of experiments had been carried out to obtain good separations of the above-mentioned cations.

Our purpose was to prove that the differences of reduction potential can be used to obtain chromatographic separation of ions; therefore our experiments were carried out principally from that point of view.The influence of other factors, such as potential gradient on the electrode, concentration and volume of the solution, liquid flow rate and differences of reduction potentials, on the quality of the chromatographic separation were limited, in order to find the values that provide separation.

We found that the quality of the separation is not influenced by the resistance of the graphite electrode in the range 0.5–40 Ω. However, for electrodes of electric resistance less than 10 Ω noticeable heating of the electrode occurred, which caused evolution of gas bubbles and consequently the layers of deposited metal were not uniform. For this reason we only used electrodes with a higher resistance than 10 Ω.

Because of the short distance, limited by the length of the graphite electrode, we had to use small amounts of solution varying from 0.1 to 0.5 ml (0.5 ml in the case of two-component mixtures when the reduction potential differences were significant) with a higher concentration than 0.2 *N*. The salt concentrations were so chosen that even layers of deposit were obtained, which adhered to the electrode satisfactorily, thus facilitating visual control of the process.

The layers of deposited metals were very rough when the concentrations of cations in the solution were less than 0.2 *N*. This can be attributed to the vigorous evolution of hydrogen in such conditions. When the concentration was higher than 1 *N* and the flow rate was 1 ml/h, no separation of two-component mixtures was achieved, even if the difference in their reduction potentials was significant. The silver ion concentration was less than 0.2 *N* when silver was separated from copper in sulphuric acid because of the low solubility of silver sulfate. At lower concentrations of sulphuric acid, the quality of the copper layer was insufficient. The silver layers so obtained were comparatively good because the reduction potential of this metal is much more positive than that of hydrogen.

It is evident that the rate of the liquid flowing out of the column is closely

TABLE I

Separated cations	*Composition of solution*	*Flow rate*	*Supporting liquid*	*Appearance of the chromatogram*	*Methods of analysis*	*Conclusions*
Ag^+ Cu^{++}	$AgNO_3$ 0.02 g/ml $CuSO_4$ 0.08 g/ml H_2SO_4 0.01 g/ml	1 ml/h	CCl_4	White uneven layer of Ag, graphite, reddish white layer of Cu followed by red layer of Cu, gradually becoming black	Visual and quantitative after scraping off a part of the precipitate	Successive separation of Ag and Cu. Pure layers of Ag and Cu without mixed layers
Ag^+ Cd^{++}	$AgNO_3$ 0.02 g/ml $Cd(NO_3)_2$ 0.10 g/ml NH_4OH 0.02 g/ml	3 ml/h	$CHCl_3$ CCl_4	White layer of Ag, graphite, black layer of Cd (not typical)	Visual	Successive separation of Ag from Cd in separate layers
Cu^{++} Zn^{++}	$CuSO_4$ 0.08 g/ml $ZnSO_4$ 0.08 g/ml NH_4OH 0.04 g/ml	2.2 ml/h	$CHCl_3$ $C_2H_5OC_2H_5$	Red-black layer of Cu, graphite, grey-white layer of Zn	Visual	Successive separation of Cu from Zn in two separate layers
Cd^{++} Zn^{++}	$CdCl_2$ 0.09 g/ml $ZnSO_4$ 0.08 g/ml NH_4OH 0.04 g/ml	2 ml/h	CCl_4	Typical grey layer of Cd, graphite, typical white-grey layer of Zn	Visual, spectral	Successive separation of Cd from Zn in two separate layers
Ag^+ Cu^{++} Cd^{++}	$AgNO_3$ 0.02 g/ml $Cu(NO_3)_2$ 0.09 g/ml $Cd(NO_3)_2$ 0.09 g/ml NH_4OH 0.20 g/ml	4 ml/h	CCl_4	White layer of Ag, graphite, red-black layer of Cu, black layer of Cd (not typical)	Visual	Successive separation of Ag, Cu and Cd in three separate layers
Cu^{++} Cd^{++} Zn^{++}	$CuSO_4$ 0.08 g/ml $CdCl_2$ 0.09 g/ml $ZnSO_4$ 0.08 g/ml NH_4OH 0.20 g/ml	2.4 ml/h	$CHCl_3$ $C_2H_5OC_2H_5$	Red-black layer of Cu, dark brown layer of Cd, graphite, grey-white layer of Zn	Visual, spectral	Existence of pure layers. Complete separation of all three cations: Cu, Cd, Zn
Ag^+ Cu^{++} Cd^{++} Zn^{++}	$AgNO_3$ 0.02 g/ml $Cu(NO_3)_2$ 0.10 g/ml $Cd(NO_3)_2$ 0.07 g/ml $Zn(NO_3)_2$ 0.09 g/ml NH_4OH 0.20 g/ml	4 ml/h	$CHCl_3$ $C_2H_5OC_2H_5$	White layer of Ag, graphite, uneven, red-black layer of Cu, dark brown layer of Cd, graphite, white-grey layer of Zn	Visual	Successive separation of Ag, Cu, Cd and Zn in four separate layers

Experimental conditions: volume of solution: 0.2 ml; potential difference on the graphite electrode: 3.3 V; electrodes: platinum and graphite of 18 Ω resistance.

connected with the concentration and volume of the solutions being examined. For the ranges of volume and concentration given above the flow rate was maintained between 1–6 ml per hour. Attempts to separate cations with reduction potentials that differ only slightly, such as nickel and cadmium in ammoniacal solution, failed because the chromatographic column used was not long enough.

The appearance of the chromatogram was the most important factor when choosing the conditions of separation. Only uniform and well-adhering layers of deposited metal on the electrode would enable one to draw quantitative conclusions. The layers obtained in our work were often uneven and for that reason we made no attempt to draw any quantitative conclusions from the appearance of the chromatograms. Since we failed to work out the composition of an electrolyte that would permit chromatographic separation of all the cations, we limited ourselves to electrolytes with compositions suitable for groups of some cations. For example with an acid solution it is possible to separate ions that have more positive reduction potentials than hydrogen.

Only the ions that form complexes with ammonia and whose hydroxides dissolve in ammonia solutions were separated in this environment. Similarly, with potassium cyanide we only attempted to separate cations that are able to form complex cyanide ions. However, it appeared that with cyanide solutions the adhesion of the layers is not sufficient to give a satisfactory separation of ions.

SUMMARY

A new method of chromatography based upon the differences in the reduction potentials of metals has been proposed.

The apparatus applied in electrolytic chromatography is described and data on the separation of some cations by this method are given.

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SÉPARATIONS DE *p*-BENZOQUINONES NATURELLES PAR CHROMATO-PLAQUES

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La présence de *p*-benzoquinones dans les sécrétions des invertébrés, est assez répandue. (Pour une revue plus complète sur cette question, voir réf.[1,2].) Nous avons précédemment montré[1], la présence de *p*-benzoquinones dans les sécrétions de Myriapodes. *Spirostreptus castaneus attems* contient la *p*-benzoquinone, *Spirostreptus virgator silvestri* et *Pachybolus laminatus*, contiennent la méthyl-*p*-benzoquinone. Ces substances ont été isolées sous forme d'hydroquinones, après entraînement à la vapeur effectué sur les extraits, puis réduction par l'hydrosulfite de sodium. Nous avons décrit alors l'étude par chromatographie sur papier de ces hydroquinones, ainsi que celle d'une série d'hydroquinones utilisées comme témoins. SCHILDKNECHT[3] a obtenu de bonnes séparations des *p*-benzoquinones du coléoptère *Brachynus crepitans*, en effectuant la chromatographie sur papier des 2,4-dinitrophénylhydrazones. GASPARIČ[4] a décrit le comportement de la *p*-benzoquinone par chromatographie sur papier. Il ne semble pas que la chromatographie sur papier des dérivés de la *p*-benzoquinone ait été déjà décrite.

Dans une revue d'ensemble[5], DEMOLE a montré l'intérêt considérable que présentait pour l'étude des substances naturelles, la technique des chromato-plaques. Cette technique permet une investigation rapide, utilisant peu de matériel; elle est susceptible d'être appliquée à de nombreuses familles de substances[6,7].

Nous décrivons l'utilisation des chromato-plaques pour la recherche des *p*-benzoquinones.

Ayant reçu sept espèces nouvelles de Myriapodes, mais en quantités insuffisantes pour permettre l'isolement des hydroquinones, nous avons entrepris la recherche des *p*-benzoquinones sur plaques d'acide silicique. Nous pensons que la technique chromatographique sur plaques, peut être particulièrement utile dans la recherche préliminaire, systématique, de ces quinones. Par cette méthode, nous avons pu montrer que les sept espèces de Myriapodes que nous avons reçues, contenaient toutes la méthyl-*p*-benzoquinone. Il semblerait que cette substance soit plus répandue chez ces animaux, que la *p*-benzoquinone elle-même. Les espèces étudiées sont les suivantes: en provenance d'Abidjan: *Peridontopyge vachoni**, *Peridontopyge aberrans attems*, *Aulonopygus aculeatus barbieri*, *Aulonopygus aculeatus attems*, ainsi que deux espèces non identifiées; en provenance de Boukoko: *Spirostreptus multisulcatus demange.*

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* Nous remercions le Professeur VACHON, et J. M. DEMANGE, laboratoire de Zoologie du Museum d'Histoire Naturelle, Paris, pour l'identification de ces animaux.

La méthode des chromatoplaques s'applique également à l'analyse des *p*-benzoquinones de structure plus compliquée. Nous avons obtenu dans les mêmes conditions que précédemment, une bonne séparation de la 2,5-dihydroxy-3-*n*-undécyl-*p*-benzoquinone[*] (Embeline isolée de *Embelia ribes*) et de la 2-méthyl-5,6-diméthoxy-*p*-benzoquinone (voir Fig. 1). Avec le système benzène–acétate d'éthyle (4:1), il est possible d'obtenir des R_F convenables pour les quinones plus polaires que la *p*-benzoquinone. Nous avons ainsi pu comparer une 2,6-diméthoxy-*p*-benzoquinone, isolée de l'écorce de *Simaruba amara* par POLONSKY ET LEDERER[9], à la substance authentique[**] (voir Fig. 2).

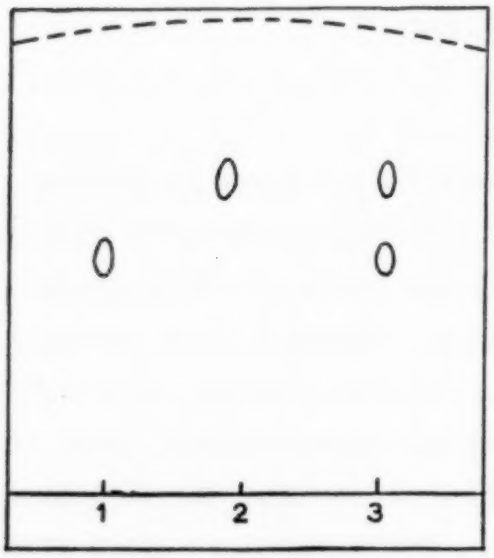

Fig. 1. Système *n*-hexane–acétate d'éthyle (85:15). (1) 2-Méthyl-5,6-diméthoxy-*p*-benzoquinone, R_F 0.47. (2) Embeline, R_F 0.62. (3) Mélange 1 + 2.

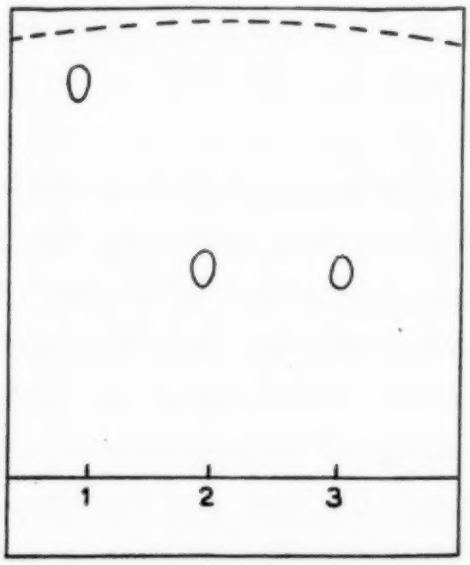

Fig. 2. Système benzène–acétate d'éthyle (4:1). (1) *p*-Benzoquinone, R_F 0.85. (2) 2,6-Diméthoxy-*p*-benzoquinone isolée de *Simaruba amara*, R_F 0.44. (3) 2,6-Diméthoxy-*p*-benzoquinone authentique, R_F 0.44.

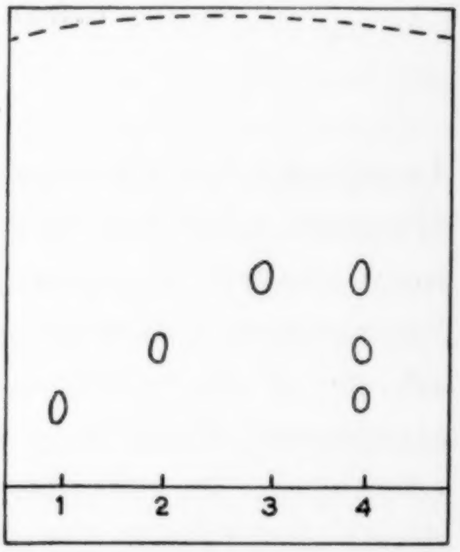

Fig. 3. Système *n*-hexane–acétate d'éthyle (85:15). (1) *p*-Benzoquinone, R_F 0.21. (2) Méthyl-*p*-benzoquinone, R_F 0.43. (3) Éthyl-*p*-benzoquinone, R_F 0.58. (4) Mélange 1 + 2 + 3.

Il est nécessaire d'utiliser un témoin à chaque essai, les conditions décrites ne permettant pas une reproductibilité parfaite des R_F.

Technique utilisée

On broye dans un mortier, un ou deux Myriapodes, sous une faible couche d'éther. L'éther se colore en jaune et doit être séparé aussitôt des débris d'animaux; on peut vérifier par un test de réduction (hydrosulfite de sodium ou anhydride sulfureux) que la coloration obtenue est bien due à des quinones. On réalise immédiatement les taches sur la plaque. A cet effet, des plaques de pyrex de 11.5/17 cm ont été enduites du mélange d'acide silicique (Mallinckrodt) et d'amidon, selon la technique décrite par REITSEMA[8] et DEMOLE[5]. L'activation des plaques par chauffage à 90–105° durant une heure, n'a pas conduit à de bonnes séparations des substances témoins (*p*-benzoquinone, méthyl-*p*-benzoquinone, éthyl-*p*-benzoquinone); les quinones sont dans ce cas trop fortement retenues par l'adsorbant. Nous avons trouvé que cette séparation était réalisable, à condition d'opérer sur des plaques d'acide silicique inactivé. Il suffit pour cela, de retirer les plaques du four dès que la couche est suffisamment durcie (environ 10 minutes) et de les abandonner ensuite 48 heures à l'air. Nous avons utilisé comme système de développement, le mélange *n*-hexane–acétate d'éthyle (85:15). On obtient dans ces conditions d'excellentes séparations (voir Fig. 3).

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[*] Nous remercions le Dr. O. SCHINDLER, Institut de Chimie Organique de l'Université de Bâle, pour les échantillons nécessaires à cet essai.

[**] Nous remercions Mme POLONSKY et le Professeur LEDERER, pour l'échantillon isolé de *Simaruba amara*, et le Professeur MARINI-BETTÒLO (Rome), pour l'échantillon authentique.

Bibliographie p. 651.

REMERCIEMENT

Nous remercions le Professeur E. LEDERER, Paris, pour l'intérêt qu'il a sans cesse montré pour ce travail.

RÉSUMÉ

La séparation de *p*-benzoquinones, par chromatographie sur plaques d'acide silicique est décrite. Cette technique s'applique à l'étude directe des *p*-benzoquinones naturelles, isolées de sécrétions animales, ou bien de végétaux.

SUMMARY

The separation of *p*-benzoquinones on plates of silicic acid is described.This technique can be used to investigate *p*-benzoquinones isolated from animal or plant secretions.

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FLAVYLIUMPERCHLORAT ALS REAGENS IN DER PAPIERCHROMATOGRAPHIE

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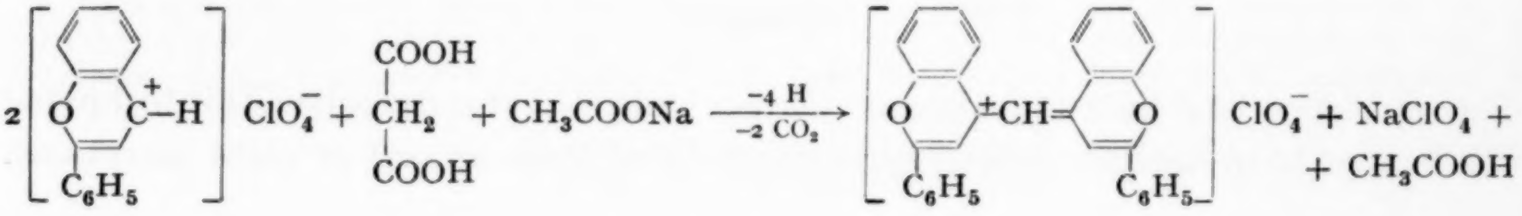
Flavyliumperchlorat (F) gibt nach einer Untersuchung von Bartos[1] mit verschiedenen organischen Verbindungen in essigsaurer Lösung Farbreaktionen von teilweise sehr hoher Empfindlichkeit. Es erschien daher interessant, die Verwendbarkeit dieser Reaktion zum Nachweis organischer Substanzen auf Papierchromatogrammen zu prüfen.

F bildet mit einer Reihe von Stoffen intensiv gefärbte Verbindungen. Nach Wizinger[2] kommen hier vor allem Substanzen in Frage, die aromatische Amino-, beziehungsweise reaktionsfähige Methylen- oder Methylgruppen enthalten. Wizinger gibt folgendes Reaktionsschema (am Beispiel der Malonsäure) an:



Bartos[1] führte die Farbreaktionen mit einer 0.25%igen essigsauren F-Lösung aus, die noch 2% Na-acetat zur Pufferung enthielt. Dabei wurde die zu untersuchende Substanz in 5 ml reiner Essigsäure gelöst und mit 0.5 ml des oben genannten Reagens 30 Minuten im siedenden Wasserbad erwärmt. Unter diesen Bedingungen konnte von manchen Substanzen noch 1 μg nachgewiesen werden. Die Färbungen gehorchten innerhalb mittlerer Extinktionsbereiche dem Beer'schen Gesetz. Hohe Empfindlichkeit wurde besonders bei Malonsäure, Indol und aromatischen Aminen mit freier *p*-Stellung erreicht. Eine weitere Reihe von Substanzen (zum Beispiel alizyklische Ketone, gewisse Phenole) führten zu schwächeren Färbungen, während Alkohole, Aldehyde, Säuren, Ester, Zucker, aliphatische Amine und Aminoaldehyde keine brauchbaren Reaktionen lieferten.

Um zu klären, ob die von Bartos[1] in Lösung durchgeführten Farbreaktionen auch auf dem Papier vor sich gehen, haben wir zunächst eine Reihe von Substanzen auf Chromatographiepapier gebracht. Die Verbindungen wurden in einer Menge von 10 μg in geeigneter Lösung so aufgetragen, dass der Durchmesser der Flecken etwa 15 mm betrug. Beim Entwickeln mit F konnten bei verschiedenen Stoffen nun tatsächlich Farbreaktionen beobachtet werden. Da beim blossen Auftropfen auf Papier andere Bedingungen als bei der Wanderung in Lösungsmitteln vorliegen, haben wir diejenigen Substanzen, die unter den oben genannten Bedingungen deutliche Farbreaktionen ergeben hatten, mit geeigneten Lösungsmitteln wandern gelassen und mit F entwickelt. In allen Fällen wurden 10 μg Substanz verwendet, da dieses Quantum den in der Papierchromatographie üblicherweise verwendeten Mengen entspricht.

METHODIK

1. Das F wurde nach der Vorschrift von WIZINGER[2], ausgehend von Salicylaldehyd und Acetophenon, hergestellt.
2. Als Reagens verwendeten wir eine 0.25%ige Lösung von F in Eisessig. Auf einen Zusatz von Na-acetat wurde verzichtet, da unter unseren Versuchsbedingungen kein Einfluss dieses Zusatzes zu beobachten war.
3. Die Papierchromatogramme wurden möglichst gleichmässig kräftig mit dem Reagens besprüht und anschliessend etwa 5 Minuten lang im Trockenschrank auf 105° erwärmt.
4. Die Betrachtung der Chromatogramme erfolgte sowohl im Tages- wie auch im UV-Licht (Philips-Analysen-UV-Lampe).

Die Papierchromatogramme zeigten nach Entwickeln mit F im Tageslicht einen leicht grünlichen Farbton, im UV-Licht erschienen sie hellrot. Da die Färbungen im sichtbaren Licht verhältnismässig schwach und wenig charakteristisch waren, werden in Tabelle I nur die Farben im UV-Licht angegeben.

TABELLE I

Substanz	*Farbe im UV-Licht nach Entwickeln mit F*
Adrenalin	gelb-braun
Nor-adrenalin	gelb-braun
5-Hydroxytryptamin	gold-gelb
Histamin	keine Färbung
Orcin	blau-grau
Gallussäure	keine Färbung
Pyrogallol	blau-grün
Brenzkatechin	blau-grün
Phloroglucin	blau
Hydrochinon	bräunlich
p-Aminosalicylsäure	blau-grau
Indol	blau-rot
Skatol	grau-violett
m-Toluylendiamin	rot-violett
a-Naphtylamin	keine Färbung
m-Phenylendiamin	keine Färbung
o-Phenylendiamin	keine Färbung
Benzidin	keine Färbung
Indolessigsäure	grau-blau
Malonsäure	blau
Bernsteinsäure	keine Färbung
Milchsäure	keine Färbung

Wie aus Tabelle I zu ersehen ist, sind die Ergebnisse bei der Anfärbung von Papierchromatogrammen mit F ähnlich den von BARTOS[1] angegebenen Farbreak-

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tionen in Lösungen. So ist auch hier beispielsweise der Nachweis von Malonsäure besonders empfindlich. Es ist möglich, die sonst in Papierchromatogrammen schwer nachzuweisende Malonsäure nicht nur in geringen Mengen sichtbar zu machen, sondern auch von anderen Säuren zu unterscheiden; so zeigt die nächsthöhere Dikarbonsäure—Bernsteinsäure—keine Farbreaktion mit F. Dieses spezielle Verhalten von Malonsäure und Bersteinsäure könnte vielleicht so erklärt werden, dass im Falle der Bernsteinsäure die Methylengruppen weniger reaktionsfähig sind als die der Malonsäure; gerade diese sind aber nach den Angaben von WIZINGER[2] für das Zustandekommen der Farbreaktion mit F von Bedeutung.

Bei Einteilung der Farbreaktionen der einzelnen untersuchten Substanzen nach ihrem Intensitätsgrad, ergibt sich ungefähr folgende Reihung:

(a) Besonders intensive Färbungen lieferten:
- Malonsäure
- Indol
- Pyrogallol
- *m*-Toluylendiamin
- Indolessigsäure

(b) Schwächere Färbungen ergaben:
- Adrenalin
- 5-Hydroxytryptamin
- Brenzkatechin
- Phloroglucin
- Hydrochinon
- Skatol

(c) Nur angedeutet färbten sich:
- Nor-adrenalin
- Orcin
- *p*-Aminosalicylsäure

Die oben angeführten Stichproben gestatten die Annahme, dass die Anwendung von F als Sprühreagens bei manchen papierchromatographischen Fragestellungen von Nutzen sein kann.

ZUSAMMENFASSUNG

Die Brauchbarkeit von Flavyliumperchlorat in der Papierchromatographie wurde gezeigt. Die Reaktionsprodukte einer Reihe von Substanzen mit Flavyliumperchlorat wiesen im sichtbaren Licht, besonders aber im UV-Licht Färbungen auf, die in einigen Fällen sehr spezifisch waren.

SUMMARY

The applicability of flavylium perchlorate in paper chromatography was demonstrated. The reaction products of a number of substances with flavylium perchlorate showed colours in visible light and especially in ultraviolet light. In some cases the colours were highly specific.

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Eingegangen den 25. März 1959

SHORT COMMUNICATIONS

The analysis of isoprene by gas chromatography

Of

Ziegler Catalyst polymerization studies required an analytical method for possible poisons in isoprene, such as cyclopentene, cyclopentadiene and acetylenic compounds. This paper gives retention data for these, and other hydrocarbons, on two of the gas–liquid chromatographic columns which have been of use in this work. The liquid phases used were dimethyl sulfolane (DMS) and benzyl cyanide–silver nitrate (BC–$AgNO_3$).

BC–$AgNO_3$ has been used by VAN DE CRAATS[1] and others, but no retention data have been published. DMS is a commonly used liquid phase. KNIGHT[2] shows the separation of a pentene blend with DMS in the form of a chromatogram. No retention data have been published for C_5 acetylenes on DMS or the effect of temperature on the relative retention times.

Apparatus. A Beckman GC-2 with an external water bath for controlling the temperature to ± 0.05° was used. The carrier gas was helium and the detector temperature 31°. The 18 ft. DMS column used was of 1/4 in. copper and contained 8.62 g of DMS on 43.08 g of Johns Manville 30–50 mesh Chromosorb. The 11.4 ft. BC–$AgNO_3$ column was of 1/4 in. stainless steel and contained 3.806 g of silver nitrate dissolved in 9.660 g of benzyl cyanide on 33.31 g of the above chromosorb. The liquid phase was added directly to the solid support and mixed by stirring in each case.

The rate of flow of the carrier gas was measured by timing the displacement of water from a 500 ml gas burette and correcting to the column temperature and outlet pressure. The column inlet pressure was measured with a mercury manometer at the liquid injection point. The carrier gas pressure gauge indicates the pressure at the inlet side of the capillary orifices, and does not indicate the pressure on the chromatographic column.

Discussion. The method of presentation of data in Tables I and II was recommended by AMBROSE, KEULEMANS AND PURNELL[3]. The same symbols are used. Relative retention times are reported at three different temperatures and partition coefficients were measured in duplicate at each temperature.

Nomenclature

V_s = volume of solvent at the column temperature (ml)
z = distance between air and solute peaks (in.)
p_g = carrier gas gauge pressure (p.s.i.g.)
p_i = carrier gas pressure at column inlet (mm/Hg)
p_o = carrier gas pressure at column outlet (mm/Hg)
F_c = carrier gas flow rate corrected to column temperature and outlet pressure (ml/min)
U = recorder chart speed (1 in. per min)
t_x = distance between start and solute peak (in.)
w = width of peak as measured at the base-line intercepts of the tangents to the peak (in.)
f = pressure gradient factor $= \dfrac{3\,(p_i/p_o)^2 - 1}{2\,(p_i/p_o)^3 - 1}$
K = partition coefficient $= \dfrac{\text{weight of solute per ml of solvent}}{\text{weight of solute per ml of gas}} = \dfrac{F_c f z}{V_s U}$
n = the number of theoretical plates $= 16\left(\dfrac{t_x}{w}\right)^2$

TABLE I

RETENTION DATA FOR HYDROCARBONS ELUTED FROM DIMETHYL SULFOLANE

Hydrocarbon	*Relative retention times*					
	35.0°		*22.25°*		*15.0°*	
Acetylene	0.137		0.116		0.106	
Isopentane	0.217		0.200		0.184	
n-Pentane	0.284		0.263		0.247	
3-Methylbutene-1	0.294		0.260		0.244	
Butadiene-1,3	0.369		0.329		0.311	
Neohexane	0.382		0.359		0.343	
Propyne	0.384		0.356		0.350	
Pentene-1	0.414		0.393		0.378	
2-Methylbutene-1	0.482		0.461		0.447	
trans-Pentene-2	0.503		0.486		0.476	
cis-Pentene-2	0.551		0.530		0.518	
Pentadiene-1,4	0.615		0.592		0.574	
2-Methylbutene-2	0.634		0.618		0.608	
n-Hexane	0.706		0.698		0.692	
Cyclopentane	0.756		0.717		0.712	
Butyne-1	0.800		0.789		0.774	
Isoprene	1.00		1.00		1.00	
Isopropylacetylene	1.055		1.075		1.096	
Cyclopentene	1.075		1.04		1.04	
2-Methylpentene-1	1.094		1.108		1.115	
trans-1,3-Pentadiene	1.335		1.358		1.375	
Butyne-2	1.41		1.435		1.456	
cis-1,3-Pentadiene	1.48		1.514		1.541	
n-Heptane	1.724		1.823		1.887	
Cyclopentadiene	1.90		1.954		1.995	
Pentyne-1	1.96		2.09		2.18	
Isopropenylacetylene	2.21		2.38		2.50	
Pentyne-2	2.66		2.86		3.01	
V_s	7.69		7.60		7.55	
z	13.10	13.27	12.43	12.72	26.15	26.92
p_g	26.2		42.0		26.2	
p_i	1225	1221	1533	1526	1192	1206
p_o	738	739	741	746.5	734	749
F_c	54.85	52.97	102.9	100.5	52.09	51.25
f	0.891	0.894	0.708	0.717	0.913	0.922
K for isoprene	83.27	81.73	119.1	120.6	164.6	168.5
n for isoprene	2595		2127		2195	

Density of dimethyl sulfolane = 1.1362 g/c.c. at 20° ± 0.001°.

Most hydrocarbon impurities (C_5 and less) can be determined if both chromatographic columns are used. If the BC-$AgNO_3$ column alone is used, the α-acetylenes are not determined, as these react with the silver nitrate. Since the relative retention times alter as the liquid phase changes composition, the column packing requires changing more often than with conventional liquid phases. This column is good, however, for the detection of butyne-2 when 1,3-pentadiene is present (both are common impurities in isoprene). For the DMS analysis, it is of interest to note that the resolution between isoprene and cyclopentene is best at the higher temperature, while the resolution between isoprene and isopropylacetylene is improved at lower temperature.

TABLE II

RETENTION DATA FOR HYDROCARBONS ELUTED FROM BENZYL CYANIDE–SILVER NITRATE

Hydrocarbon	*Relative retention times*					
	30.0°		*22.0°*		*15.0°*	
n-Butane	0.041		0.036		0.034	
Ethylene	0.042		0.041		0.038	
Propylene	0.100		0.095		0.091	
n-Pentane	0.111		0.103		0.093	
Propadiene	0.115		0.103		0.094	
trans-Butene-2	0.154		0.1465		0.136	
Isobutylene	0.195		0.1875		0.180	
n-Hexane	0.268		0.254		0.246	
Butene-1	0.287		0.287		0.284	
cis-Butene-2	0.345		0.330		0.327	
Cyclopentane	0.367		0.342		0.325	
Butadiene-1,3	0.366		0.353		0.336	
trans-Pentene-2	0.371		0.355		0.352	
2-Methylbutene-2	0.393		0.381		0.366	
3-Methylbutene-1	0.452		0.457		0.457	
2-Methylbutene-1	0.598		0.602		0.603	
Pentene-1	0.597		0.609		0.626	
n-Heptane	0.649		0.633		0.638	
Cyclohexane	0.811		0.766		0.750	
cis-Pentene-2	0.810		0.832		0.851	
Isoprene	1.00		1.00		1.00	
2-Methylpentene-1	1.122		1.155		1.194	
trans-1,3-Pentadiene	1.377		1.390		1.407	
Butyne-2	1.619		1.608		1.606	
Pentadiene-1,4	1.571		1.648		1.735	
cis-1,3-Pentadiene	1.825		1.874		1.924	
Cyclopentadiene	2.464		2.460		2.50	
Cyclopentene	2.461		2.611		2.778	
Pentyne-2	3.236		3.353		3.490	
V_s	10.50		10.42		10.36	
z	14.60	14.17	11.42	11.49	15.43	15.30
p_g	30		48		48	
p_l	1061	1059	1267	1267	1263	1253
p_o	745	746	736.6	745.8	750	746
F_c	72.26	72.20	138.7	135.3	132.1	132.1
f	1.064	1.070	0.858	0.870	0.878	0.880
K for isoprene	107	104	130.5	129.7	172.7	171.7
n for isoprene	1122		990		998	

Density of benzyl cyanide = 1.015 g/c.c. at 18° ± 0.001°.
Density of silver nitrate = 4.352 g/c.c. at 19°.

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The paper electrophoresis of amphoteric elements and their complexes with polyhydroxy compounds in KOH

The paper electrophoretic separation of inorganic anions with KOH or NaOH as electrolyte was investigated by JACH *et al.*[1] and GRASSINI AND LEDERER[2]. The last-mentioned authors also examined solutions of Cu(II) and other metals in KOH and found that although a number of amphoteric elements are readily soluble in KOH, they do not move anionically in paper electrophoresis. These results were not reported.

An ion exchange separation of Cu and Cd in NaOH using glycerol as complexant for Cu(II) was recently described by KRESHKOV AND SAYUSHKINA[3]. It thus occurred to us that the absence of anionic movement of amphoteric anions may be due to complexing with the cellulose of the paper.

We have investigated this question by carrying out electrophoretic experiments under the following conditions:

1. Electrophoresis on Whatman No. 1 cellulose filter paper with *N* KOH as electrolyte.
2. As in (1) but with *N* KOH containing 1% glycerol.
3. Electrophoresis on Whatman glass fibre paper with *N* KOH as electrolyte.
4. As in (3) with KOH containing 1% glycerol.

The results with Sb(III), Sn(II), Al(III), Zn(II), Cu(II), Pb(II), and Bi(III) are shown in Table I. Fe(III), Zr(IV), Ni(II), Co(II), Rh(III), Rh(IV) and Ti(IV) did not move in any of the four conditions.

TABLE I

MOVEMENT OF METAL IONS IN 2 HOURS WITH 250 V USING A GLASS PLATE TECHNIQUE WITH PAPERS 30 cm LONG

Metal ion	*Electrolyte and paper as in*			
	1	*2*	*3*	*4*
Bi(III)	0	0	0	22
Pb(II)	0	4	37	34
Cu(II)	0	0	0	42
Zn(II)	61	72		72
Al(III)	30	53		53
Sn(II)	28	25		62
Sb(III)		0		22
Po(IV)	0			0

Bismuth does not move except on glass paper in presence of glycerol. It thus seems that bismuthite does not migrate, but a glycerol complex does unless hindered by the cellulose complex formed on cellulose paper. Cu(II) and Sb(III) behave similarly.

Lead does not move on cellulose but moves on glass paper in presence and absence of glycerol. It thus appears that an anionic plumbite exists in solution and is only retarded on paper by the formation of a (probably unstable) cellulose complex.

Since the presence of glycerol on glass paper does not change its mobility, it is impossible to confirm the existence of a glycerol complex except by the slight movement exhibited on cellulose in presence of glycerol and the probability of its existence if a cellulose complex forms.

Al(III), Zn(II) and Sn(II) migrate on cellulose and glass fibre in presence and absence of glycerol. However, on glass fibre in presence of glycerol there is a faster movement suggesting that there may be competition between a weak cellulose complex and a stronger glycerol complex. Polonium showed very slight tailing forward when subjected to electrophoresis on glass paper in presence of glycerol. Since we are dealing here with tracer concentrations, conclusions are difficult.

We have presented evidence that complex formation between cellulose and amphoteric ions must be taken into account when paper chromatography or paper electrophoresis is attempted in strongly alkaline medium.

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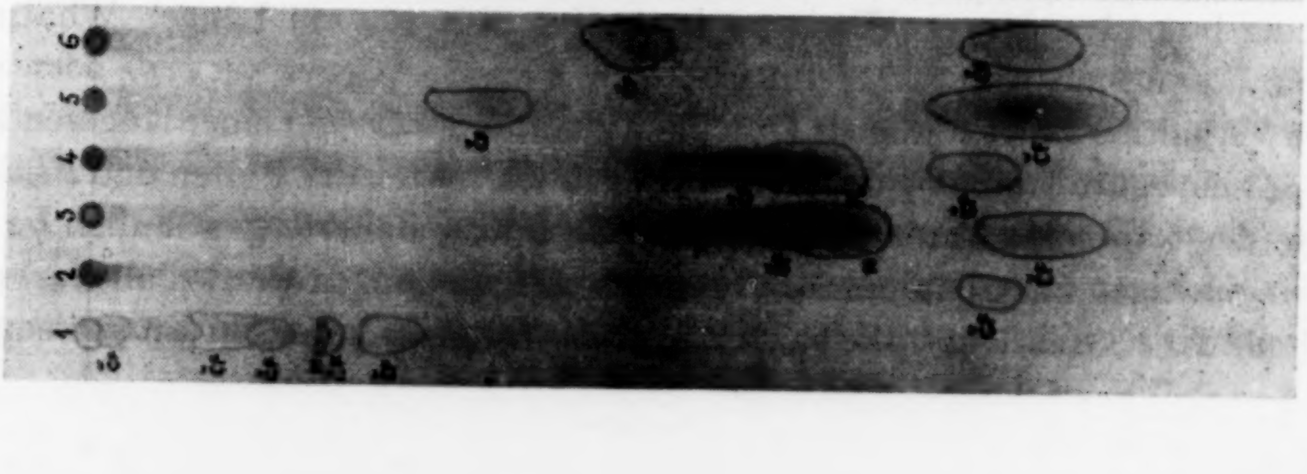
Zur Papierchromatographie der Pflanzenfarbstoffe

Im Rahmen der Forschung bezüglich des Gehalts der Pflanzenfarbstoffe in den Blüten verschiedener Pflanzenarten entstand die Notwendigkeit, eine grössere Anzahl von papierchromatographischen Analysen der methanolischen Extrakte der Pflanzenblüten durchzuführen. Bisherige Methoden der papierchromatographischen Analyse der Pflanzenfarbstoffe, besonders der Anthocyane, geben gute Ergebnisse oft erst nach einer vorläufigen Reinigung der Extrakte[1]. Da jedoch die Reinigung der Extrakte oft zu erheblichen Verlusten an Pflanzenfarbstoffen führt, versuchten wir eine Methode der papierchromatographischen Analyse der Extrakte zu finden, die eine gute Trennung der Pflanzenfarbstoffe, besonders der Anthocyane, auch in den ungereinigten Extrakten ermöglichen würde. So haben wir festgestellt, dass bei der Anwendung besonders langer Filtrierpapierstreifen (bis 100 cm Länge) Whatman No. 1 im Lösungsmittelsystem *n*-Butylalkohol–Eisessig–Wasser (4:1:5, oder viel besser im Verhältnis 10:1:3) mittels Durchlaufverfahren während einer längeren Zeit (bis 100 St.) eine gute Trennung der Anthocyane in Extrakten der Blüten und Früchte erzielt werden kann. Die chromatographische Trennung dauerte gewöhnlich 72 bis 100 St., in günstigen Fällen nur 48 St. Unter diesen Bedingungen wurden alle farbigen Bestandteile der Extrakte über die ganze Länge des Chromatograms in gut getrennten



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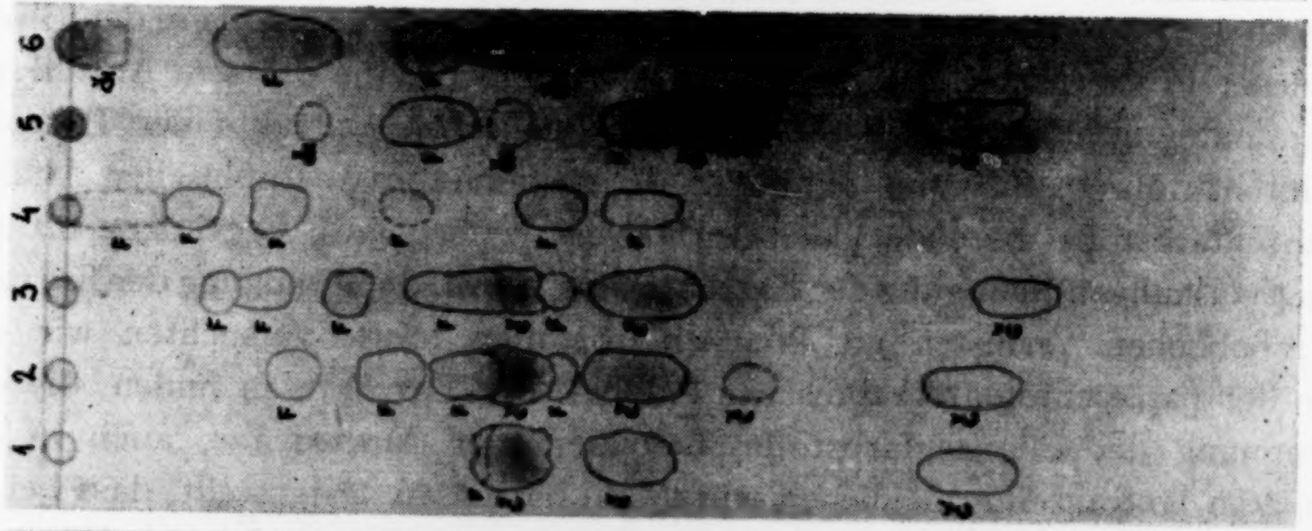

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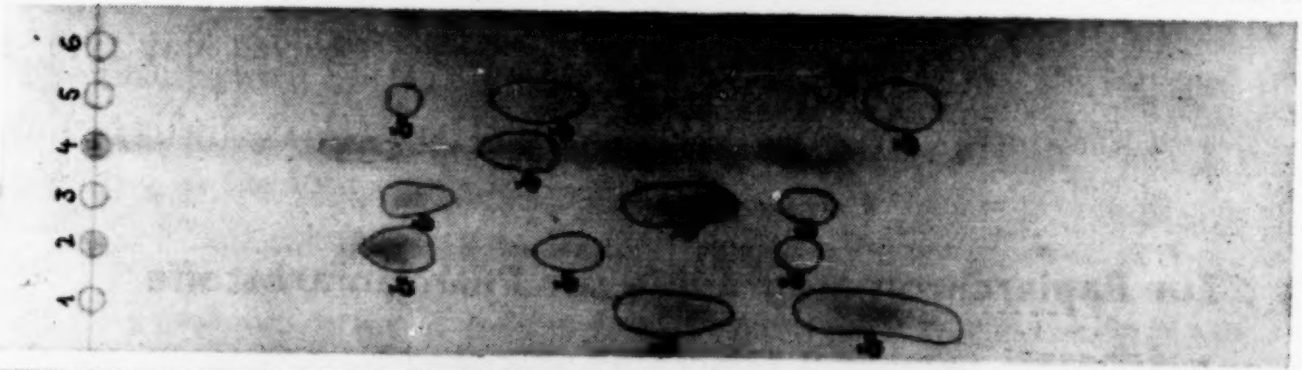

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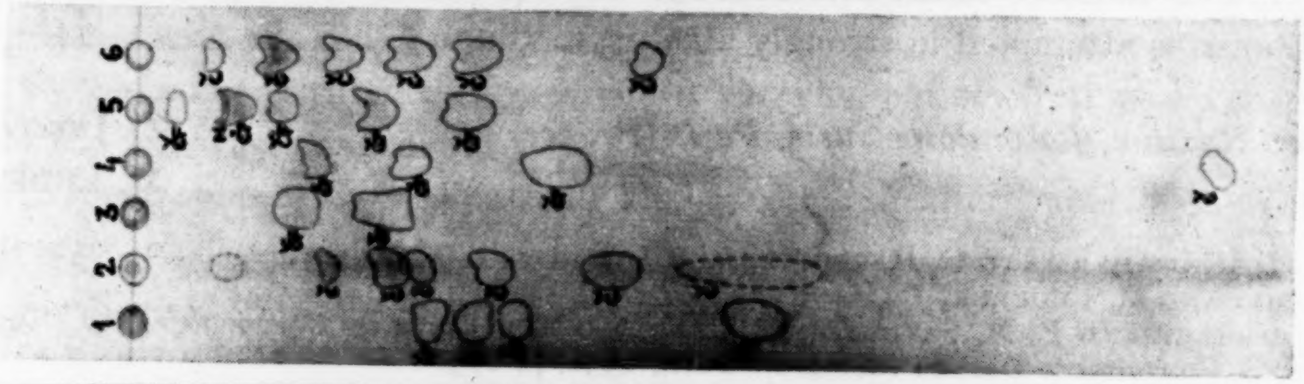

Fig. 4.

Fig. 5.

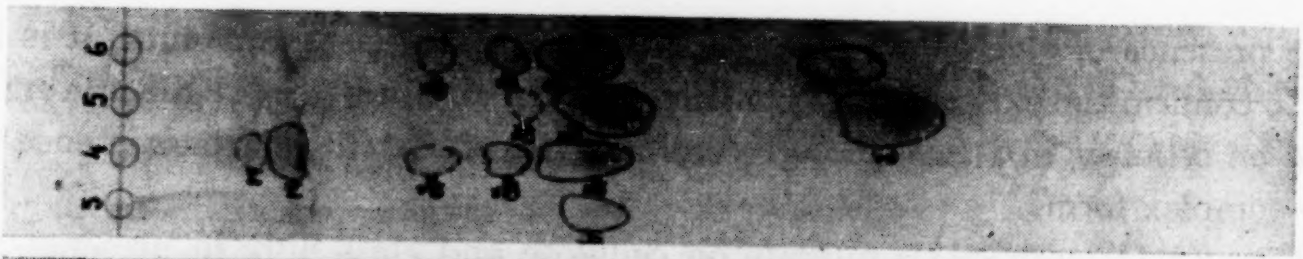

Fig. 6.

Farben Abkürzungen: R = rot; F = violett; M = blau; ČF = rot-violett; MF = blau-violett; OR = orangerot; Ž = gelb.

Flecken verteilt. In der Mischung *n*-Butylalkohol–Eisessig–Wasser (10:1:3) dauerte das Entwickeln der Chromatogramme längere Zeit, die Flecken der Farbstoffe waren aber voneinander gut getrennt und bildeten keine "Schwänze".

In einigen Fällen, zum Beispiel bei den Extrakten aus den Blüten der Glöckchen (*Campanula*) hat sich für die chromatographische Trennung der Anthocyane die von uns neu vorgeschlagene Mischung *n*-Propylalkohol–3 % HCl (65:35) besser bewährt als die oben erwähnte Mischung von *n*-Butylalkohol–Eisessig–Wasser.

Die papierchromatographische Analyse der Anthocyane auf den langen Filtrierpapierstreifen Whatman No. 1 gab bessere Ergebnisse als die Durchlaufchromatographie auf den Streifen Whatman No. 1 normaler Länge (etwa 55 cm); auf dem kürzeren Papier sind die Flecke ziemlich dicht beisammen und voneinander zu wenig getrennt. Mehrfaches Entwickeln hat sich bei der Analyse der Anthocyane nicht bewährt; die Flecken verblassten allmählich um endlich ganz zu verschwinden.

Die Ergebnisse der chromatographischen Analyse der Blüten und Früchte verschiedener Pflanzenarten werden in den Figuren 1–10 veranschaulicht. Auf diese Weise wurde auch der Einfluss des Trocknens auf den Anthocyan-Gehalt in den Blüten der Heilpflanzen verfolgt.

Auf die Chromatogramme, die im Lösungsmittelsystem *n*-Butylalkohol–Eisessig–Wasser (10:1:3) entwickelt wurden, wurden kleinere Mengen der Extrakte aufgetragen, so dass in einigen Fällen die sehr kleinen Mengen einiger Anthocyane nicht erfasst wurden. Auf die Chromatogramme, die in der Mischung *n*-Butylalkohol–Eisessig–Wasser (4:1:5) entwickelt wurden, wurden grössere Mengen der Extrakte aufgetragen, was die Erfassung auch der Spurenbestandteile ermöglichte. Die Identifizierung der Anthocyane wird erst nach der Beschaffung der notwendigen Standardpräparate durchgeführt werden.

Die Technik des langzeitigen oder mehrfachen Entwickelns der Chromatogramme auf den 100 cm langen Filtrierpapierstreifen Whatman No. 1 bewährte sich gut auch bei der Analyse der Aminosäuren in den Extrakten aus Pflanzen- und Tierengeweben sowie in den Hydrolysaten der Eiweisstoffe; auch bei der Analyse der Zuckergemische

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Fig. 1. Lösungsmittelsystem *n*-Butylalkohol–Eisessig–Wasser (10:1:3), absteigend. Extrakte aus den Blüten bzw. Früchten der Pflanzen. Extrakte: 1. *Consolida ajacis*; 2. *Antirrhinum majus*; 3. *Antirrhinum majus*; 4. *Antirrhinum red chief*; 5. *Scabiosa atropurpurea*; 6. *Ribes silvestre* (Früchte).

Fig. 2. Lösungsmittelsystem wie bei Fig. 1. 1. *Gladiolus* (rot); 2. *Gladiolus* (rot-violett); 3. *Gladiolus* (dunkelrot); 4. *Brassica oleracea var. capitata*; 5. *Begonia*; 6. *Sambucus nigra* (Früchte).

Fig. 3. Lösungsmittelsystem wie bei Fig. 1. 1. *Althaea armeniensis*; 2. *Begonia* (rosa) 3. *Begonia saepeflorens*; 4. *Begonia bulbosa*; 5. *Pelargonium zonale*; 6. *Pelargonium*.

Fig. 4. Lösungsmittelsystem wie bei Fig. 1. 1. *Dabaecia*; 2. *Matthiola annua*; 3. *Clarkia pulchella*; 4. *Iris–Kuma Kura*; 5. *Ipomoea aitoni* (blaue Blüten); 6. *Ipomoea* (rote Blüten).

Fig. 5. Lösungsmittelsystem wie bei Fig. 1. 1. *Papaver somniferum* (Opiumart, Bulgarien; Rand der Blütenhülle); 2. *Papaver somniferum* (Opiumart, Bulgarien; Blütenhülle); 3. *Papaver somniferum* (Dětenický bílosemenný; Rand der Blütenhülle); 4. *Papaver somniferum* (Dětenický bílosemenný; Blütenhülle); 5. *Papaver somniferum* (Dunajský modrý; Rand der Blütenhülle); 6. *Papaver somniferum* (Dunajský modrý; Blütenhülle).

Fig. 6. Lösungsmittelsystem wie bei Fig. 1. 3. *Paeonia*; 4. *Callistephus chinensis*; 5. *Rosa*; 6. *Cosmos bipinnatus*.

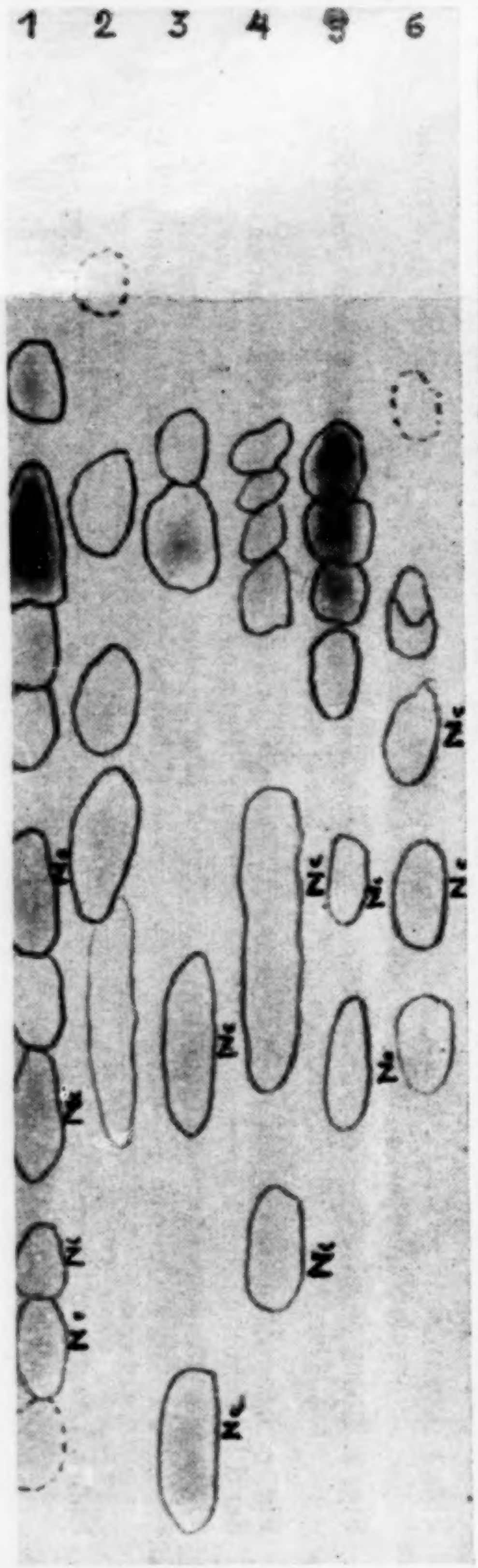

Fig. 7.

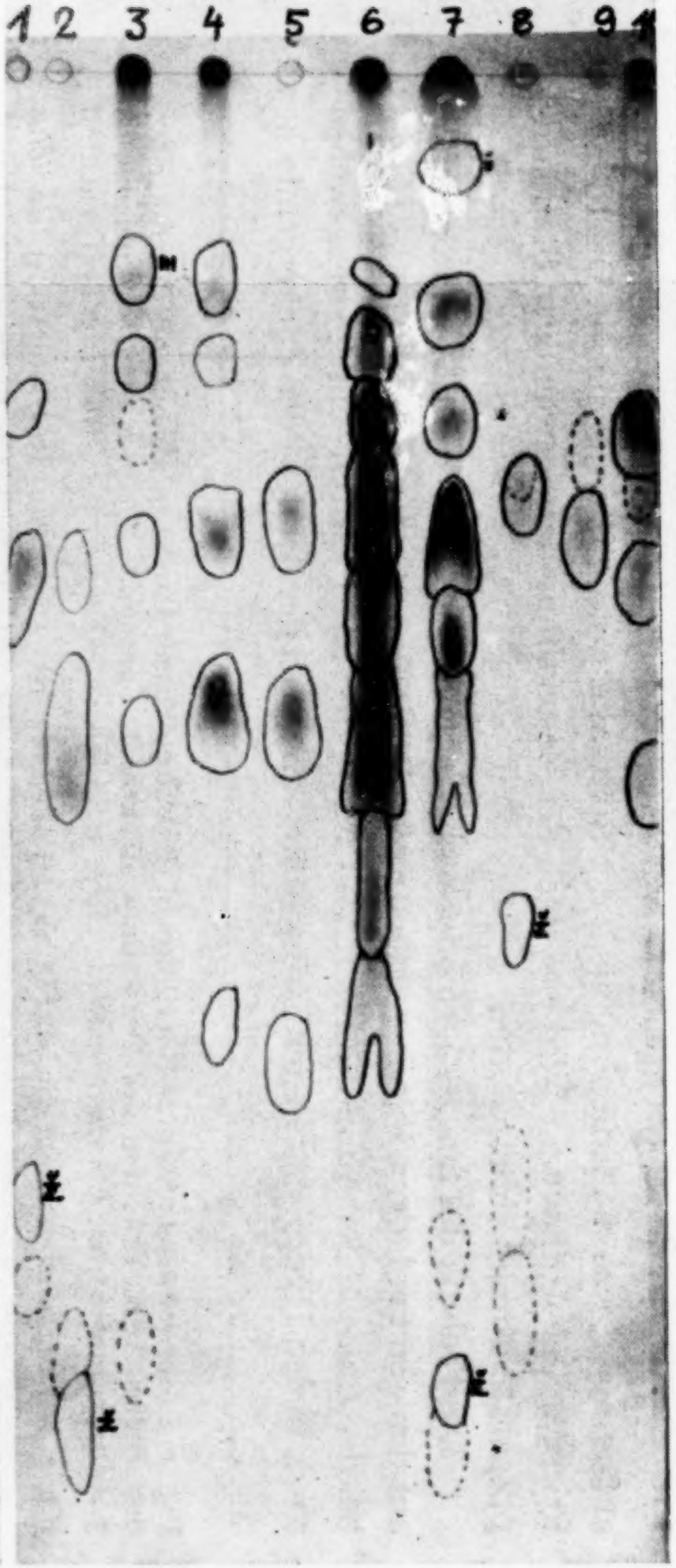

Fig. 8.

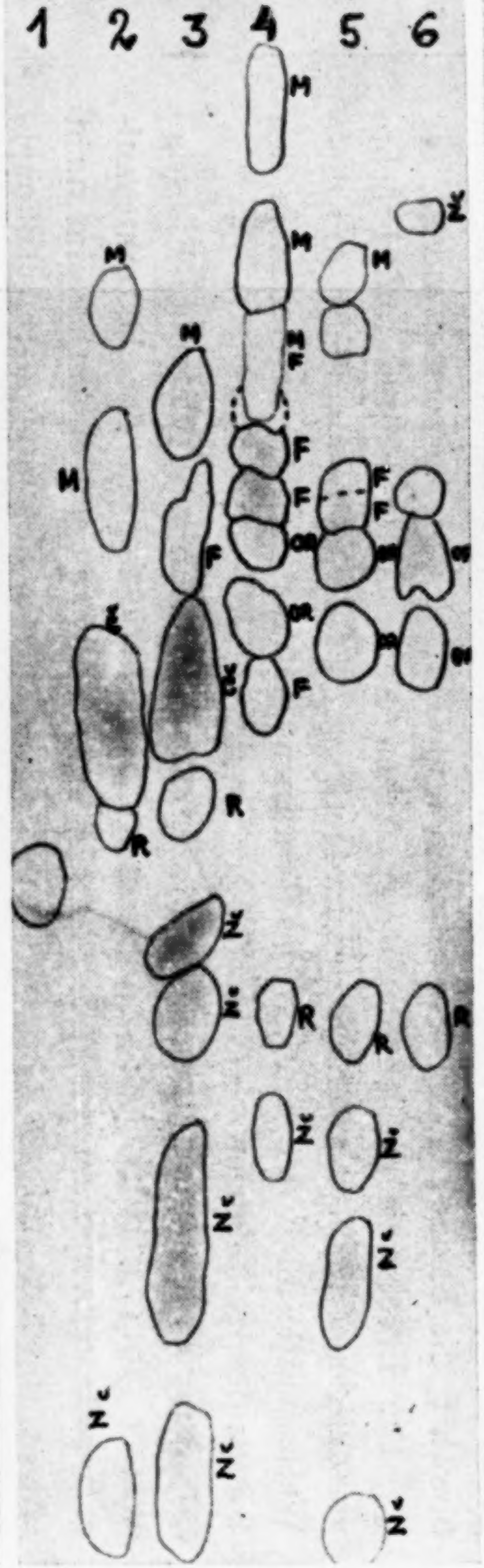

Fig. 9.

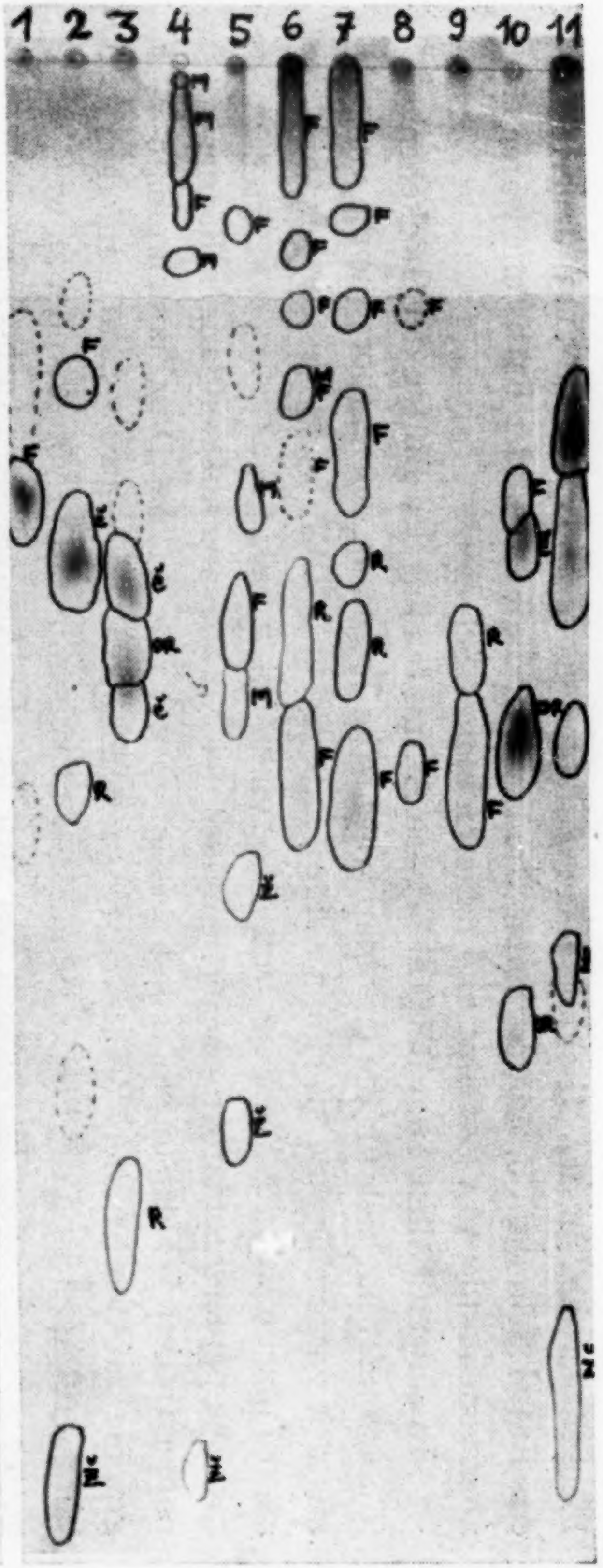

Fig. 10.

Farben Abkürzungen: R = rot; F = violett; M = blau; ČF = rot-violett; MF = blau-violett; OR = orangerot; Ž = gelb.

in biologischem Material wurde die Technik des mehrfachen Entwickelns auf den 100 cm langen Filtrierpapierstreifen erfolgreich angewandt. Auf diese Weise können auch andere Stoffe, synthetische und natürliche, im geeigneten Lösungsmittelgemisch erfolgreich getrennt werden, sogar besser als mit der üblichen Entwicklungstechnik. Die ziemlich lange Dauer des Entwickelns wird hier durch eine bessere, schärfere Trennung der Stoffe in den untersuchten Proben überwogen. Die Flecken der getrennten Stoffe sind in der Regel genügend weit voneinander entfernt, so dass eine Durchführung der quantitativen Auswertung der Chromatogramme (fotometrisch *in situ* oder kolorimetrisch in den Eluaten der Flecken aus dem Papier) ermöglicht wird. Ein weiterer Vorteil dieser Technik beruht auf der Tatsache, dass die Menge der Probe, die eindimensional analysiert werden soll, auf dem langen Papierstreifen grösser sein kann, als auf Streifen normaler länge. Eine gute Trennung wird auch dann erzielt, wenn etwa die Hälfte oder sogar zwei Drittel der Menge, welche normalerweise zweidimensional analysiert wird, auf das Papier aufgetragen wird. So können auch bei eindimensionalem Verfahren Stoffe, die bloss in Spuren anwesend sind, gut erfasst werden. Bei dieser Technik könnte auch bei der Anwendung des Filtrierpapiers Whatman No. 3 eine grössere Menge der Probe analysiert werden und dadurch auch die präparative Darstellung der Spurenbestandteile bei eindimensionalem Entwickeln erleichtert werden.

Die grossen, langen Papierstreifen können absteigend oder horizontal entwickelt werden.

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Fig. 7. Lösungsmittelsystem *n*-Butylalkohol–Eisessig–Wasser (4:1:5). Extrakte aus Blüten bzw. Früchten. 1. *Scabiosa atropurpurea*; 2. *Salvia coccinea*; 3. *Althaea armeniensis*; 4. *Nymphaea rubra*; 5. *Begonia*; 6. *Viola tricolor* (rot).

Fig. 8. Lösungsmittelsystem wie bei Fig. 7. 1. *Paeonia*; 2. *Althaea rosea*; 3. *Papaver rhoeas* (alte getrocknete Blüten); 4. *Papaver rhoeas* (getrocknete Blüten); 5. *Papaver rhoeas*; 6. *Vaccinium myrtillus* (Früchte); 7. *Sambucus nigra* (Früchte); 8. *Malva silvestris* (getrocknete Blüten); 9. *Malva silvestris*; 10. *Rosa* (rot).

Fig. 9. Lösungsmittelsystem wie bei Fig. 7. 1. *Althaea nigra*; 2. *Althaea armeniensis*; 3. *Althaea rosea*; 4. *Gladiolus* (dunkelrot); 5. *Gladiolus* (rot); 6. *Gladiolus* (orangerot).

Fig. 10. Lösungsmittelsystem wie bei Fig. 7. 1. *Malva silvestris*; 2. *Paeonia*; 3. *Pelargonium*; 4. *Consolida ajacis*; 5. *Viola tricolor*; 6. *Papaver somniferum* (Opiumart, Bulgarien); 7. *Papaver somniferum* (Dětenický bílosemenný); 8. *Papaver somniferum* (Hatvanyi, Ungarn); 9. *Papaver somniferum* (Dunajský modrý); 10. *Papaver rhoeas*; 11. *Rosa* (rot).

σ~ column silica gel

Einfluss des Säulenmaterials bei Gasadsorptionschromatographie auf Kieselgel

Die sep'n Trennung der HC Kohlenwasserstoffe C_1–C_3 auf Kieselgel wurde schon mehrfach bearbeitet[1–3]. Vorteilhaft auf die Trennung und die Lebensdauer des Kolonnen haben sich sogen. "Tailing reducer" erwiesen[4]. Bei Verwendung von Kieselgelen verschiedener origin Herkunft wurden unterschiedliche Trenneigenschaften festgestellt, welche auf eine unterschiedliche preparation Herstellung des Adsorptionsmaterials schliessen lassen. Die Analysen wurden mit einem Fraktometer Modell 116 der firm Firma Bodenseewerk Perkin-Elmer & Co. G.m.b.H., Überlingen/Bodensee durchgeführt. Die in Tabelle I gezeigten Werte wurden mit 2 m Kolonnen, vom Durchmesser 4.75 mm, bei 50° und Helium als Trägergas (Durchfluss 50 ml/min) bestimmt. Als Adsorptionsmaterial wurden

(a) Kieselgel für chromatographische Zwecke, Sorte Em, Firma Schuchart, München,

(b) Kieselgel für chromatographische Zwecke, Sorte Ee, Firma Schuchart, München,

(c) Kieselgel amerikanischer Herkunft,

miteinander verglichen. Die graining Körnung war in allen Fällen 0.15–0.30 mm; das Adsorptionsmaterial wurde mit 3% Dioctylsebacinat als "Tailing reducer" coverage belegt.

Die in Tabelle I enthaltenen relativen retaining volumes Rückhaltevolumina (Propan = 1) sind bis auf das Acetylen recht ähnlich. Das Acetylen wird aber auf dem Kieselgel Em gut vom Propan und Propylen getrennt, während es auf dem Ee Material wenig und auf dem Kieselgel (c) überhaupt nicht getrennt wird. Ausser der guten Trennung des Acetylens shows weist das Em Kieselgel auch das kleinste Rückhaltevolumen für Propan auf, wodurch die Analysen auf diesem Material sehr accelerated beschleunigt werden. In Fig. 1 ist die Trennung der Kohlenwasserstoffe von C_1–C_5 auf einer 4 m Säule reproduced wiedergegeben.

TABELLE I

RÜCKHALTEVOLUMINA FÜR KOHLENWASSERSTOFFE C_1–C_3

(a) relativ zu Propan = 1.
(b) korrigierte Rückhaltevolumen für Propan.

	Kohlenwasserstoffe	*Kieselgel Em*	*Kieselgel Ee*	*Kieselgel (c)*
(a)	Methan	0.04	0.03	0.02
	Äthan	0.27	0.28	0.23
	Äthylen	0.50	0.44	0.38
	Propan	1.00	1.00	1.00
	Acetylen	1.34	1.1[illegible]	1.00
	Propylen	2.26	1.91	2.43
(b)	V° Propan	95.4 ml	174 ml	292.1 ml

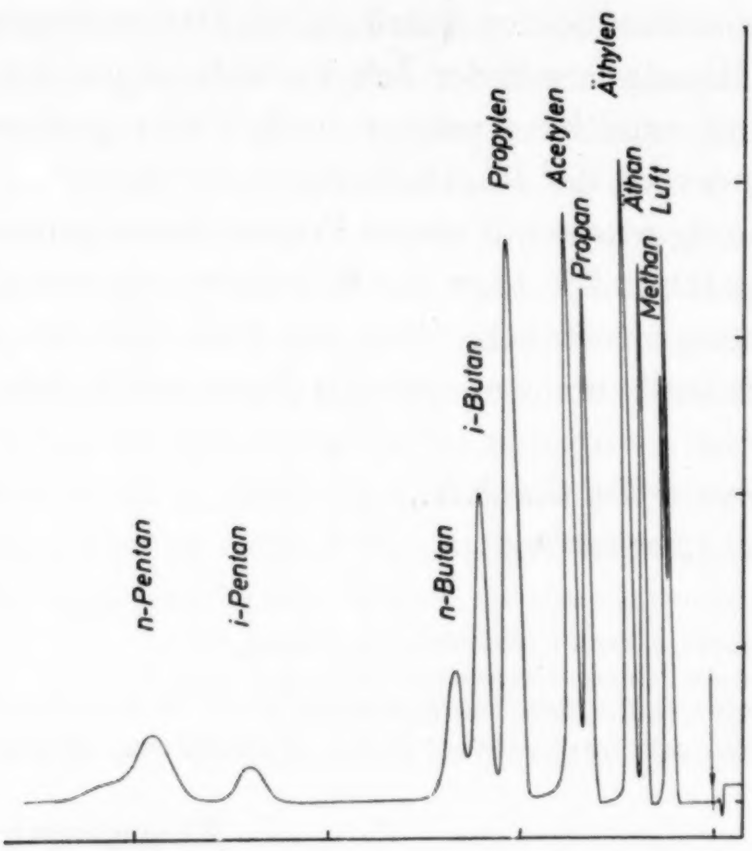

Fig. 1. Trennung C_1–C_5 Kohlenwasserstoffe. Säule: Kieselgel Sorte Em, 0.15–0.30 mm + 3% Dioctylsebacat; 4 m; Temperatur 50°. Durchfluss 40.2 ml/min Helium.

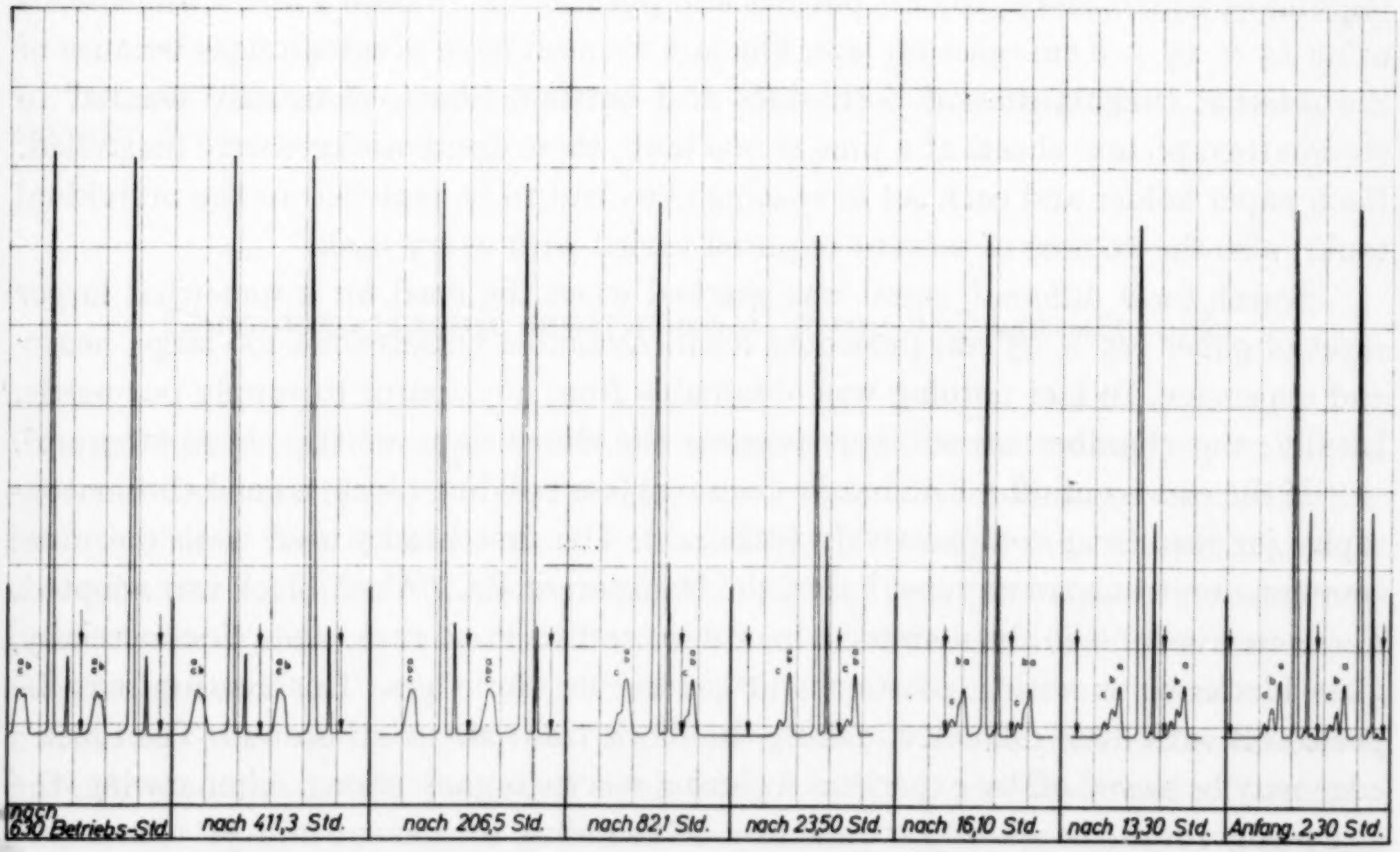

Fig. 2. Stabilität einer Kieselgel-Säule. Trennung eines Propan–Butangemisches. Arbeitsbedingungen wie Fig. 1; Recordervorschub verkleinert.

Die Stabilität der einzelnen Säulen wurde durch Dauerversuche geprüft. Dabei zeigte es sich, dass alle Kieselgele mit der Zeit Veränderungen der Bandenlage verzeichnen. Dieser Umstand muss bei Analysen in Betracht gezogen werden. Fig. 2 zeigt die langsame Veränderung der Rückhaltezeiten der einzelnen Banden auf dem Kieselgel Em. Die Trennung wurde mit einem Propan–Butangemisch über eine Zeit von 620 Stunden durchgeführt. Die Lage der Banden wurde mit a, b, c, bezeichnet und ist aus der Abbildung ersichtlich. Über die Ursachen des unterschiedlichen Verhaltens des Kieselgele wird zu einem späteren Zeitpunkt berichtet.

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[1] J. JANAK, *Collection Czechoslov. Chem. Communs.*, 19 (1954) 700.
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Glass building blocks as chromatography jars*

We have run, by the ascending technique, hundreds of 13 × 13 cm paper chromatograms of amino acids[1], sugars, purines and pyrimidines in both 1 and 2 dimensions using 15 × 15 × 8 cm museum jars. The jars we used have disadvantages because of the internal irregularities of both sides and bottom. Since we usually wanted to chromatograph four sheets at a time in one tank, these disadvantages were magnified. Each paper holder and each set of spacing clips had to be matched to the individual tanks, also the volume of solvent required varied with every tank.

Search for a different vessel was sparked when the need for a somewhat larger sheet of paper (25 × 25 cm) presented itself. Available vessels were too large, heavy and expensive. In fact nothing was obtainable from any source to supply our needs. Ideally, the chamber should approximate the dimensions of the chromatogram[2].

If the end is cut off a Pittsburgh Corning glass building block, a good chromatography jar results at comparatively little cost. The first blocks used were the more common non-transparent type. Later, the transparent P.C. "Vue" block was adopted. These are available in the nominal 8 in. × 8 in. and 12 in. × 12 in. sizes. Commercially, glass blocks come with a plastic bond coating on the edges. This coating may be peeled off with little difficulty making the block transparent all around. The block's edge may be sawed off by experienced glazing service organizations. After sawing, the sharp edges must be slightly rounded by belt sanding to prevent injury.

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* Finished jars with suitable fittings may be obtained from Arthur H. Thomas Co., 3rd and Vine Streets, Philadelphia 5, Pa., U.S.A.

"Mortite"* is applied to the lip of the jar to provide a seal and to hold in place glass rods 3 mm in diameter that support the sheets of paper. Pieces of polyethylene tubing about 2 cm long and 3 mm diameter are slit lengthwise for use as clips to attach the paper to the rods. Two clips per rod are sufficient support. If two or three sheets are used per tank no spacers are required; however, if four or five sheets are run simultaneously spacers are needed. The spacers can readily be made of polyethylene (or similar material) by cutting evenly spaced slits 4–5 mm deep into 1.5 mm(1/16 in.)thick strips 10–12 mm wide by 78 mm long. An alternate method of fastening the paper to the rod is by folding the upper edge of the paper over the rod and attaching it by clips made of 1.5 mm (1/16 in.) thick 13 mm square polyethylene. A slit 5–6 mm long is cut into the square. The cut ends of the clip are twisted, parallel to the cut, to ease the insertion of the paper and then twisted in the opposite direction to clamp the paper together.

Solvent is introduced to the bottom of the jar by a long-stemmed funnel, care being taken not to drip any solvent onto the chromatograms. The lid is then pressed into place.

The 12 in. × 12 in. block tanks may be equipped with troughs, supports, etc. for descending chromatography.

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* "Mortite" is a caulking putty made by J. W. Mortell Co., Kankakee, Ill., and obtainable in hardware stores.

Chromatographic separation of some Δ^4-3-ketosteroids

In a previous report it was shown that oestrogens could be successfully separated by adsorption chromatography on the partially esterified cation exchange resin Amberlite IRC 50[1]. Since then it has been shown that lower fatty acids can be separated on a non-esterified column of the same resin[2]. This finding prompted the author to investigate the separation of non-aromatic steroids on a partially esterified cation exchanger and the separation of some of the common Δ^4-3-ketosteroids has now been achieved.

About 50 ml of Amberlite IRC 50 (H form, pulverized and screened as described earlier[2]) was boiled with 500 ml of a mixture of ethanol, methanol and 2 N hydrochloric acid (15:5:6 by vol.) for 40 hours. This partially esterified resin was transferred to a glass filter with a solvent composed of ethanol, methanol and water (15:5:11 by vol.). After washing with the solvent, the resin was suspended in 2 volumes of the same solvent. The suspension was poured into a chromatographic tube and allowed to settle under gravity. A column 0.8 cm in diameter and 108 cm in height was used. The steroids were dissolved in the same solvent as that used for the packing of the column and 1 ml of the solution was placed on the column. After the solution had drained into the column, the inner wall of the chromatographic tube was washed with 0.1 to 0.2 ml of the solvent and the steroids were eluted with the same solvent. The effluent was collected in fractions of 40 drops in test tubes graduated at 3.5 ml. The flow rate was 1 drop per minute and the ambient temperature was 10° to 15°. Each fraction was diluted to 3.5 ml with 70% (v/v) ethanol and the ultraviolet absorption was measured at 240 mμ using a Beckman DU quartz spectrophotometer (Fig. 1).

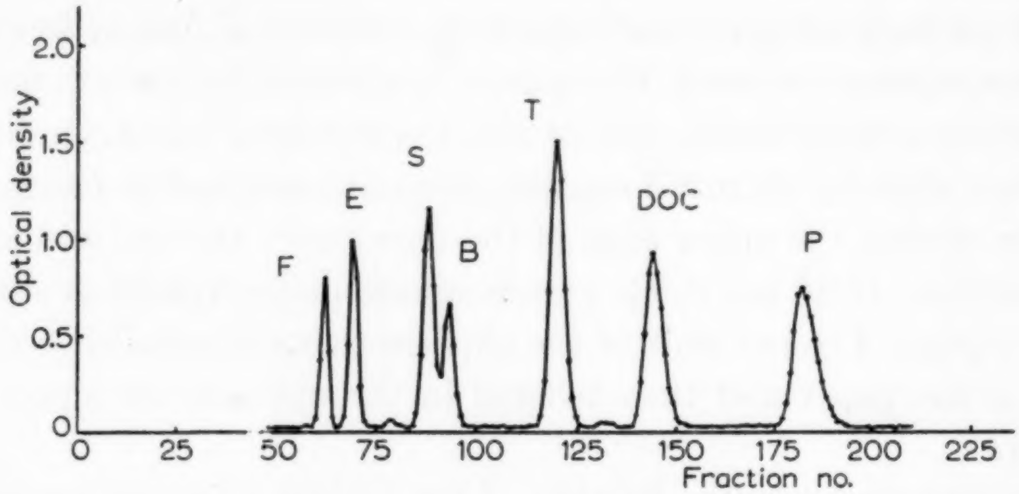

Fig. 1. Elution curve of Δ^4-3-ketosteroids. The compounds in the order of their elution from the column, are: 17-hydroxycorticosterone (F), 11-dehydro-17-hydroxycorticosterone (E), 11-desoxy-17-hydroxycorticosterone (S), corticosterone (B), testosterone (T), 11-desoxycorticosterone (DOC) and progesterone (P).

The elution sequence was similar to that of reversed phase partition chromatography, the most polar component being eluted first[3]. As shown in Table I, the recovery from the chromatographic column was satisfactory and the elution pattern was reproducible. Since the condition of the column is unchanged after chromatography, the column can be used repeatedly.

TABLE I

RECOVERY OF Δ^4-3-KETOSTEROIDS FROM THE CHROMATOGRAPHIC COLUMN

Steroids	*Added (µg)*	*Recovered (µg)*	*Recovery (%)*
17-Hydroxycorticosterone	150	134	89
11-Dehydro-17-hydroxycorticosterone	228	207	91
11-Desoxy-17-hydroxycorticosterone	321	280	87
Corticosterone	176	168	95
11-Desoxycorticosterone	393	343	87
Testosterone	465	438	94
Progesterone	391	350	90

The author wishes to thank Shionogi & Co., Ltd. for their kindness in supplying the corticosteroids used in this work.

Department of Genetics, Osaka University Medical School, Osaka (Japan) TOKUICHIRO SEKI

[1] T. SEKI, *Nature*, 181 (1958) 768.
[2] T. SEKI, *J. Biochem.* (*Tokyo*), 45 (1958) 855.
[3] H. DANIELSSON, *Biochim. Biophys. Acta*, 27 (1958) 401.

Received May 22nd, 1959

BOOK REVIEW

Electrophoresis, Theory, Methods and Applications, edited by MILAN BIER, Academic Press, New York, 1959, xx + 563 pages, price $ 15.00.

Opinions seem to be divided concerning electrophoresis today. According to the preface electrophoresis has mainly to do with proteins; if this is assumed the rather vague titles of the many excellent contributions to this volume may be understood. The chapter on "Paper Electrophoresis" by WUNDERLY deals almost exclusively with serum proteins, the chapter "Zone Electrophoresis in Various Types of Supporting Media" by KUNKEL AND TRAUTMAN deals with techniques, theory and applications to proteins, this time including also such topics as haemoglobins and immunoelectrophoresis.

Under "Applications of Zone Electrophoresis" WIELAND deals with some smaller molecules. However, his contribution is confined to examples and gives no complete literature survey, although the literature on electrophoresis sadly lacks one.

Workers in paper electrophoresis will find much that is stimulating in the various chapters dealing with theory, "free" electrophoresis, acid–base equilibria of proteins etc. While all chapters are written by experts in their fields and leave nothing to be desired, the organization of the book is poor. In addition to misleading headings for the chapters, the reviewer cannot see a plan in the sequence of the topics. The claim of the preface that all major areas of application have been covered is unfortunately a gross overstatement. Continuous electrophoresis, for example, is only briefly mentioned in the chapter by WUNDERLY and again in the chapter by KUNKEL AND TRAUTMAN, but its problems are not adequately discussed or its field of application outlined.

In spite of these shortcomings this book should be on the desk of every chemist working with electrophoretic methods. As a reference book it could have been improved by a more detailed subject index which does not cite most topics under electrophoresis; however, the author index is well prepared. Only a few printing errors were noted.

MICHAEL LEDERER (Paris)

NEW BOOKS

Ionenaustauscher, Band I: Grundlagen, Struktur, Herstellung, Theorie, by F. HELFFERICH. (Verlag Chemie, Weinheim/Bergstr., 1959), 520 pages, price DM 48.—.

The Principles of Electrophoresis, by R. AUDUBERT AND S. DE MENDE. Translated from the French by A. J. POMERANS. (Hutchinson & Co. Ltd., London, 1959), viii + 142 pages, price 25 s. net.

Estudio de las proteinas del suero humano por medio de la electroforesis en papel, by JUAN A. MORALES MALVA. (Editorial Universitaria, Santiago, Chile, 1958), xix + 169 pages. (In Spanish.)

Principles and Practice of Gas Chromatography, edited by R. L. PECSOK. (John Wiley & Sons, Inc., New York, 1959), 226 pages, price $ 6.75.

Soviet Research on Lanthanides and Actinides, translations of 107 Soviet research reports. (Consultants Bureau, Inc., New York, 1959), 657 pages, price $ 65.—.

ERRATA

J. Chromatog., Vol. 2 (1959)

Page 566, line 2 from bottom, for *aspartic* read *glutamic*.

Page 567, lines 14 and 16 from top, for *aspartic* read *glutamic*.

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CHROMATOGRAPHIC DATA

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CHROMATOGRAPHIC DATA

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TABLE 1

R_F VALUES OF CHOLESTEROL AND CHOLESTERYL ESTERS ON SILICONIZED WHATMAN NO. 1 PAPER

(J. A. LABARRÈRE, J. R. CHIPAULT AND W. O. LUNDBERG, *Anal. Chem.*, 30 (1958) 1466)

	Chloroform 40 *Methanol* 60 *Water* 5 *Formic acid* 1	*Acetone* 50 *Ethanol* 50 *Water* 5 *Formic acid* 15	*Acetone* 50 *Ethanol* 50 *Water* 5
Cholesterol	0.87	0.90	0.92
Cholesteryl laurate	0.42	0.00	0.50
Cholesteryl myristate	0.38	0.00	0.43
Cholesteryl palmitate	0.33	0.00	0.36 approx.
Cholesteryl stearate	0.28	0.00	0.00
Cholesteryl oleate	0.32	0.17	0.41
Cholesteryl linoleate	0.37	0.24	0.47
Cholesteryl linolenate	0.41	0.30	0.53

TABLE 2

R_F VALUES OF PHOSPHOLIPIDS

(R. F. WITTER, G. V. MARINETTI, L. HEICKLIN AND M. A. COTTONE, *Anal. Chem.*, 30 (1958) 1624)

Paper: Whatman No. 1, circular technique (no impregnation).

Compound	*Octanol–lutidine–acetic acid 45:2.5:5*	*2,6-Dimethyl-4-heptanone–acetic acid 30:5*	*3-Methyl-2-butanone–acetic acid 30:3*	*Chloroform–lutidine–acetic acid 20:30:5*
Yeast lecithin	0.50	0.60	0.78	0.60
Distearyl lecithin	0.49	0.58	0.76	0.57
Egg cephalin	0.35	0.47	0.60	0.54
Distearyl phosphatidyl ethanolamine	0.25	0.46	0.56	0.53
Lysolecithin	0.16	0.25	0.31	0.15
Sphingomyelin	0.35	0.38	0.44	0.35

TABLE 3

R_F VALUES OF ORGANIC MERCURY COMPOUNDS

(J. KANAZAWA, K. KOYAMA, M. AYA AND R. SATO, *J. Agr. Chem. Soc. Japan*, 31 (1957) 872)

Solvent: butanol saturated with *N* ammonia.
Temperature: 30° C.

Compound	R_F
Phenylmercury acetate	0.39
Phenylmercury chloride	0.40
Ethylmercury chloride	0.27
Methoxyethylmercury chloride	0.18
Methylmercury chloride	0.17
Hg^+ and Hg^{++}	0

TABLE 4

R_F VALUES OF AMINO ACID–COPPER COMPLEXES

(M. Beck, J. Csaszar and T. Huska, *Acta Univ. Szegediensis, Acta Phys. et Chem.*, [N.S.], 4 (1958) 35)

Paper: Schleicher and Schüll 2043a.
Reagents: Ninhydrin (after destruction of the complex with EDTA) or8- hydroxyquinoline (for Cu).

Amino acid	*R_F of Cu complex in*	
	Phenol satd. with water at 27°	*o-Cresol satd. with water at 27°*
Glycine	0.59	0.36
Alanine	0.84	0.67
Alanine	0.69	0.35
Serine	0.54	0.89
Valine	0.93	0.95
Threonine	0.76	0.40
Lysine	0.54	0.00
Proline	0.95	0.98
Hydroxyproline	0.70	0.34
Arginine	0.70	0.08
Histidine	0.09	0.53
Glutamic acid	0.20	0.00
Glycyl-glycine	0.04	0.16

TABLE 5

R_F VALUES OF ε-AMINOCAPROIC ACID AND CAPROLACTAM

(K. Czerepko, *Mikrochim. Acta*, (1958) 638)

Solvent	*R_F values*	
	ε-Amino caproic acid	*Caprolactam*
Butanol–water (86:14)	0.02	0.83
Propanol–water (50:10)	0.13	0.89
Butanol–acetone–water (10:10:5)	0.13	0.88
Butanol–propanol–water (10:10:5)	0.10	0.87
Butanol–ethanol–water (10:10:5)	0.28	0.88
Butanol–methanol–water (10:10:5)	0.38	0.86
Butanol–conc. HCl–water (100:20:38)	0.43	0.89
Butanol–acetic acid–water (10:2:5)	0.49	0.87
Propanol–acetic acid–water (7:1:2)	0.64	0.86
Cyclohexane–acetic acid–water (9:2:0.5)	—	0.37
Pyridine–water (70:30)	0.47	0.79
sec.-Butanol–formic acid–water (75:15:10)	0.46	0.8–0.9
Phenol–water (80:20)	0.85	0.9–1.0

TABLE 6

PAPER ELECTROPHORESIS OF COAL TAR FOOD COLOURS

(J. CROSSLEY AND J. D. R. THOMAS, *Analyst*, 83 (1958) 462)

Apparatus: E.E.L. electrophoresis apparatus.
Electrolytes:
1. *N* acetic acid
2. pH 4 buffer: 6 ml 0.1 *N* NaOH + 750 ml 0.1 *M* monopotassium phthalate diluted to 1.5 l.
3. pH 6 buffer: 85.5 ml 0.1 *N* NaOH + 750 ml monopotassium phthalate diluted to 1.5 l.
4. pH 8 buffer: 702 ml 0.1 *N* NaOH + 750 ml monopotassium phthalate diluted to 1.5 l.
5. 1% Sodium tetraborate.
6. 0.1 *N* NH_4OH.

	Distances moved in mm					
Electrolyte	*1*	*2*	*3*	*4*	*5*	*6*
Current density mA per 5 cm	*0.6*	*1.7*	*1.7*	*2.0*	*2.0*	*1.7*
Time in hours	*2*	*1.75*	*2*	*2*	*1.5*	*2*
Erythrosine BS	0	0	0	0	9	0
Tartrazine	130	74	23	15	103	83
Indigo carmine	52	35	9	8	38	10
Ponceau MX			0	0	0 and 16	
Ponceau 4R			26	30	100	
Ponceau 3R			0	0	11	

TABLE 7

R_F VALUES OF HYDROLYSIS PRODUCTS OF ORGANOPHOSPHATE INSECTICIDES

(F. W. PLAPP AND J. E. CASIDA, *Anal. Chem.*, 30 (1958) 1622)

Paper: Whatman No. 1.

Compound	*R_F values (at 25–28° C)*	
	2-Propanol–NH_4OH 75:25	*2-Propanol-water–NH_4OH 75:24:1*
$(HO)_2P(O)OH$	0.00	0.05
$(HO)_2P(S)OH$	0.00	0.03
$(CH_3O)P(O)(OH)_2$	0.04	0.10
$(C_2H_5O)P(O)(OH)_2$	0.07	0.15
$(i\text{-}C_3H_7O)P(O)(OH)_2$	0.08	0.21
$(n\text{-}C_4H_9O)P(O)(OH)_2$	0.16	0.31
$(CH_3O)_2P(O)OH$	0.44	0.59
$(C_2H_5O)_2P(O)OH$	0.61	0.76
$(i\text{-}C_3H_7O)_2P(O)OH$	0.74	0.88
$(n\text{-}C_4H_9O)_2P(O)OH$	0.85	0.95
$(CH_3O)_2P(S)OH$	0.65	0.73
$(CH_3O)_2P(S)OK$	0.59	0.67
$(C_2H_5O)_2P(S)OH$	0.77	0.87
$(C_2H_5O)_2P(S)OK$	0.73	0.79
$(CH_3O)_2P(S)SH$	0.74	0.85
$(CH_3O)_2P(S)SK$	0.68	0.78
$(C_2H_5O)_2P(S)SH$	0.89	0.94
$(C_2H_5O)_2P(S)SK$	0.84	0.87
$(CH_3O)(HO)P(O)OC_6H_2\text{-}Cl_3\text{-}2,4,5$	0.79	0.82
$(CH_3O)(HO)P(S)OC_6H_2\text{-}Cl_3\text{-}2,4,5$	0.86	0.96

TABLE 8

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF SOME ALIPHATIC CARBONYL COMPOUNDS

(E. SUNDT AND M. WINTER, *Anal. Chem.*, 30 (1958) 1620)

Paper: Whatman No. 7 impregnated with dimethylformamide.
Solvent: cyclohexane–cyclohexene (5:3 v/v).

Substance	R_F
Glycolaldehyde	0.06
Formaldehyde	0.29
Acetaldehyde	0.36
Diacetyl (mono)	0.38
Acetone	0.44
Hex-2-en-1-al	0.61
1-Hexanal	0.67

TABLE 9

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF SOME AROMATIC COMPOUNDS

(E. SUNDT AND M. WINTER, *Anal. Chem.*, 30 (1958) 1620)

Paper: Whatman No. 7 impregnated with dimethylformamide.
Solvent: cyclohexane–cyclohexene (5:3 v/v).

Substance	R_F
Acetovanillone	0.05
p-Hydroxybenzaldehyde	0.05
p-Hydroxyacetophenone	0.07
Salicylic aldehyde	0.15
Acetoveratrone	0.18
Anisaldehyde	0.23
Cinnamaldehyde	0.34
Benzaldehyde	0.35
Acetophenone	0.42
p-Methylphenoxyacetic aldehyde	0.43
p-Propioanisolaldehyde	0.45
3-Phenylpropionic aldehyde	0.47
1-Phenyl-butan-3-one	0.54
Cuminaldehyde	0.64
Menthone	0.84
2,4-Dinitrophenylhydrazine	0.05, 0.32, 0.71

TABLE 10

R_F VALUES OF CONDENSED AROMATIC COMPOUNDS AND THEIR OXIDATION PRODUCTS

(J. Gasparič, *Mikrochim. Acta*, (1958) 681)

Compound	*Dimethylformamide–hexane*	*α-Bromonaphthalene–90% acetic acid*
Anthracene	0.91	0.15
2-Methylanthracene	0.94	0.11
9,9′-Dianthryl	0.95	0.02
Anthrone	0.64	
Oxanthrone	0.27	
Dianthraquinone	0	0.04
10,10′-Dianthranol	0	
Anthraquinone	0.73	0.25
Anthracene-1,2-quinone	0.04	0.44
Anthracene-1,4-quinone	0.51	0.35
2-Methylanthraquinone	0.81	0.13
Phenanthrene	0.92	0.15
Phenanthrenequinone	0.16	0.37
Pyrene	0.90	0.07
Pyrene-3,8-quinone	0.05	0.35
Pyrene-3,10-quinone	0.05	0.38
Fluoranthene	0.92	0.12
Dibenzanthracene	0.88	0.04
Benzopyrene	0.88	0.04
Carbazole	0.22	0.43
Acridine	0.45	0.89

TABLE 11

R_F VALUES OF SOME NAPHTHOQUINONES, HYDROXYANTHRAQUINONES AND RELATED COMPOUNDS

(J. Gasparič, *Mikrochim. Acta*, (1958) 681)

Compound	*Dimethylformamide–hexane*	*α-Bromonaphthalene–90% acetic acid*
α-Naphthoquinone	0.52	0.55
β-Naphthoquinone	0.06	0.72
1-Methylnaphthoquinone	0.72	0.43
2-Methylnaphthoquinone	0.71	0.43
1,6-Dimethylnaphthoquinone	0.85	0.36
2,6-Dimethylnaphthoquinone	0.80	0.39
2,3-Dimethylnaphthoquinone	0.83	0.33
1-Hydroxyanthraquinone	0.05	0.24
1,2-Dihydroxyanthraquinone	0.02	0.58
1,4-Dihydroxyanthraquinone	0.80	0.18
1,2,5,8-Tetrahydroxyanthraquinone	0.00	0.49
Benzanthrone	0.51	0.17
1,4-Diacetoxyanthracene	0.16	0.47
Diacetyl anthrahydroquinone	0.08	0.51

TABLE 12

R_F VALUES OF SOME QUINONES

(J. GASPARIČ, *Mikrochim. Acta*, (1958) 681)

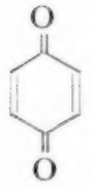
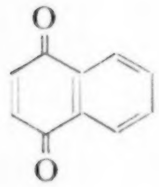
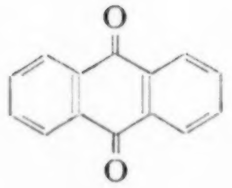
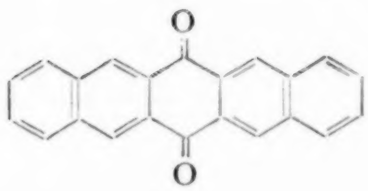
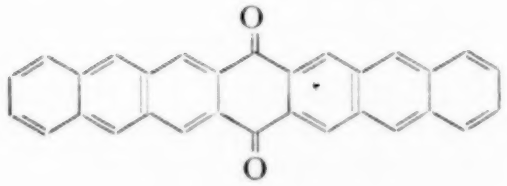
Quinone	R_F	
	Dimethylformamide–hexane	*α-Bromonaphthalene–90% acetic acid*
	0.30	
	0.52	0.55
	0.73	0.25
	0.65	0.08
	0	0.03

TABLE 13

R_F VALUES OF SOME ORGANIC ACIDS

(J. GASPARIČ, *Mikrochim. Acta*, (1958) 681)

Solvent: ethanol–ammonia–water (80:4:16).
Paper: Schleicher und Schüll 2045b.

Acid	R_F
Benzoic	0.58
Phthalic	0.28
Maleic	0.21
Anthraquinone-2-carboxylic	0.65
Naphthalene-2,3-dicarboxylic	0.33
Diphenic	0.45
Pyrenic	0.34
Naphthalene-1,4,5,8-tetracarboxylic	0.20

TABLE 14

R_F VALUES OF ALKALOID DRUGS (IN URINE)

(M. BÜHLER, *Röntgen- u. Lab.-Praxis,* 10 (1957) 284)

Solvent: butanol–formic acid–water (12:1:7).
Paper: Schleicher und Schüll No. 2043b.

Substance	R_F
Nicotine	0.23
Morphine	0.31
Dilaudid	0.32
Eucodal	0.34
Dicodid	0.39
Cliradon	0.61
Pervitin	0.65
Dolantin	0.70
Dromoran	0.71
Polamidon	0.80

TABLE 15

RELATIVE RETENTION VOLUMES OF TERPENES AND NON-CYCLIC UNSATURATED ALCOHOLS AND ESTERS (GAS CHROMATOGRAPHY)

(E. BAYER, G. KUPFER AND K.-H. REUTHER, *Z. anal. Chem.,* 164 (1958) 4)

Reference substance: camphor, V_R^{rel} = 1.00.

Substance	*Stationary phase and temperature*					
	Silicon grease C–lithium capronate		*Silicon grease C–sodium capronate*		*Silicon grease C*	
	157°	*187°*	*160°*	*185°*	*160°*	*187°*
Camphene	0.43	0.59	0.43	0.50	0.46	0.58
α-Pinene	0.55	0.62	0.51	0.595	0.63	0.59
Terpinolene	0.60	0.57	0.54	0.50	0.64	0.75
Limonene	0.65	0.75	0.57	0.595	0.70	0.64
Geranyl formate	0.58	—	—	—	—	—
Terpinyl acetate	0.78	0.99	—	—	0.825	0.87
Linalool	0.79	0.725	0.73	0.90	0.655	0.78
Fenchyl alcohol	0.95	0.915	0.885	0.90	0.61	0.69
Camphor	1.00	1.00	1.00	1.00	1.00	1.00
Citronellal	1.05	1.105	1.00	0.95	1.145	1.075
Citronellol	1.60	1.48	—	—	1.65	1.50
Carvone	1.06	1.47	1.525	1.50	1.71	1.42
Menthol	1.15	1.21	1.115	1.10	1.31	1.23
Menthone	1.065	1.07	—	1.00	1.05	1.025
Borneol	1.11	1.345	1.03	1.10	—	0.65
α-Terpineol	—	1.18	—	1.25	—	—
Citral	—	1.34	—	1.60	0.935	0.77
Geraniol	1.20	1.15	—	1.40	1.10	0.78
Bornyl acetate	—	1.54	—	—	1.78	1.445
Geranyl acetate	2.15	2.13	2.165	1.71	—	1.78
Geranyl butyrate	3.42	3.53	—	—	—	4.38

TABLE 16

RELATIVE RETENTION VOLUMES OF PHENOLS (GAS CHROMATOGRAPHY)
(J. JANAK AND R. KOMERS, *Z. anal. Chem.*, 164 (1958) 69)

Stationary phase:
I = Apiezon L.
II = Dimethyl polysiloxane.
III = Phenylmercapto-thiodiazole.
IV = Salicylideneaminoguanidine.
V = Benzyl arabinoside.
VI = γ-Lactone of galacturonic acid.
VII = Erythritol.
VIII = Dulcitol.

Substance	*Stationary phase and temperature*							
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
	170°	*170°*	*160°*	*170°*	*176°*	*150°*	*150°*	*196°*
Phenol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2-Methylphenol	1.65	1.85	1.23	0.89	1.03	0.61	0.52	0.50
3-Methylphenol	1.79	2.31	1.56	1.41	1.39	0.98	0.94	0.84
4-Methylphenol	1.75	2.30	1.55	1.41	1.36	0.97	0.88	0.83
2-Ethylphenol	2.54	—	—	—	1.25	—	0.40	—
3-Ethylphenol	2.88	—	—	—	1.99	—	0.83	—
4-Ethylphenol	2.83	—	—	—	1.97	—	0.84	—
2,3-Dimethylphenol	3.44	3.29	—	1.48	1.72	0.75	0.67	0.54
2,4-Dimethylphenol	2.90	2.90	1.85	1.20	1.34	0.56	0.47	0.46
2,5-Dimethylphenol	2.90	2.91	1.85	1.07	1.37	0.50	0.45	0.38
2,6-Dimethylphenol	2.75	2.80	—	0.92	0.78	0.35	0.23	0.30
3,4-Dimethylphenol	3.64	3.51	—	2.39	2.32	1.28	1.17	0.89
3,5-Dimethylphenol	3.16	3.58	—	1.90	1.92	0.94	0.86	0.81

TABLE 17

RELATIVE RETENTION VOLUMES OF PHENOLS (GAS CHROMATOGRAPHY)
(G. BERGMANN AND D. JENTZSCH, *Z. anal. Chem.*, 164 (1958) 10)

Stationary phase: *n*-decyl phthalate.
Temperature: 150°.
Retention volumes relative to anisole = 1.00.

Substance	*Retention volume*	*Substance*	*Retention volume*
Anisole	1.00	2,3-Dimethylanisole	3.03
Phenol	3.20	2,5-Dimethylanisole	2.57
o-Cresol	4.46	2,6-Dimethylanisole	2.00
m-cresol	5.69	3,4-Dimethylanisole	3.42
p-Cresol	5.69	3,5-Dimethylanisole	3.03
o-Ethylphenol	6.87	Phenetole	1.39
m-Ethylphenol	9.25	*o*-Methylphenetole	2.05
p-Ethylphenol	9.25	*m*-Methylphenetole	2.39
2,3-Xylenol	8.51	*p*-Methylphenetole	2.43
2,4-Xylenol	8.16	*o*-Ethylphenetole	2.23
2,5-Xylenol	7.16	*m*-Ethylphenetole	3.89
2,6-Xylenol	4.86	*p*-Ethylphenetole	4.03
3,4-Xylenol	10.42	2,3-Dimethylphenetole	4.35
3,5-Xylenol	9.41	2,5-Dimethylphenetole	3.38
o-Methylanisole	1.54	2,6-Dimethylphenetole	2.14
m-Methylanisole	1.73	3,4-Dimethylphenetole	5.30
p-Methylanisole	1.73	3,5-Dimethylphenetole	4.32
o-Ethylanisole	2.26		
m-Ethylanisole	2.80		
p-Ethylanisole	2.89		

TABLE 18

R_F VALUES OF ORGANIC PEROXIDES

(A. RIECHE AND M. SCHULZ, *Angew. Chem.*, 70 (1958) 694)

Filter paper: Schleicher and Schüll 2043 b partially acetylated.
Solvent: Ethyl acetate–dioxane–water (2:4.5:4.6).

Substance	R_F *value*
H_2O_2	0.83
Tetrahydrofuran hydroperoxide	0.76
Benzoyl peroxide	0.19
Cyclohexenyl hydroperoxide	0.46
Isochroman peroxide	0.22
Succinyl peroxide	0.82
Isochroman hydroperoxide	0.50
Cumol hydroperoxide	0.39
Lauroyl peroxide	0
Tetralin hydroperoxide	0.36
Stearoyl peroxide	0
Methylisochroman hydroperoxide	0.51
Isopropylisochroman hydroperoxide	0.36

See also *J. Chromatog. (Chrom. Data)*, 1 (1958) xx.

TABLE 19

R_F VALUES OF TROPOLONES

(C. A. WACHTMEISTER AND B. WICKBERG, *Acta Chem. Scand.*, 12 (1958) 1335)

Paper: Whatman No. 1 dipped in an aqueous solution of 0.015 *M* disodium EDTA and 0.005 *M* EDTA, dried and dipped into a 25% v/v solution of dimethylsulphoxide in toluene.

Substance	*Solvent*		
	Light petroleum b.p. 60–71°	*Cyclohexane*	*Di-isopropyl ether*
Tropolone	0.08	0.10	0.30
7-Hydroxy-4-isopropyl-tropolone	0.15	0.18	0.40
γ-Thujaplicin	0.32	0.38	0.55
β-Thujaplicin	0.40	0.45	0.60
α-Thujaplicin	0.62	0.65	0.75
Nootkatin	0.72	0.75	0.82

a.c.

TABLE

RETENTION VOLUMES OF ORGANIC COMPOUNDS RELATIVE

(G. RAUPP,

Length of column: 2 m.
Speed of carrier gas: 100 ml helium/min.

No.	*Substance*	*B.p. °C*	*A*			*B*		
			50°	*100°*	*150°*	*50°*	*100°*	*150°*
1	Isobutane	— 10.2	0.29	—	—	0.26	—	—
2	*n*-Butane	+ 0.6	0.40	0.47	0.53	0.38	0.48	0.57
3	Isopentane	31.0	0.79	0.83	1.0	0.77	0.84	1.0
4	Methyl formate	31.5	0.85	0.91	0.88	0.72	0.82	0.80
5	Furan	32.0	1.62	1.47	1.15	1.53	1.42	1.29
6	Diethyl ether	34.6	1.24	1.18	0.94	1.15	1.08	1.05
7	*n*-Pentane	36.0	1.00	1.00	1.00	1.00	1.00	1.00
8	Methylene chloride	41.6	3.17	2.69	2.38	2.97	2.33	1.94
9	Carbon disulphide	46.2	2.43	2.38	2.18	2.12	1.98	1.91
10	*n*-Propyl chloride	46.4	2.59	2.39	2.06	2.29	1.98	1.83
11	*n*-Propylamine	48.7	1.95	1.84	1.62	—	—	—
12	Propionaldehyde	48.8	2.00	1.70	1.68	1.56	1.36	1.29
13	Acrolein	52.5	2.17	1.96	1.85	1.68	1.49	1.40
14	Ethyl formate	54.1	2.16	1.91	1.80	1.91	1.64	1.40
15	Acetone	56.5	2.14	1.92	1.59	1.63	1.34	1.14
16	Methyl acetate	57.1	2.38	2.12	1.88	1.96	1.54	1.46
17	1,1-Dichloroethane	57.3	4.56	3.74	3.09	4.02	2.97	2.69
18	Chloroform	61.2	10.6	7.57	5.35	8.44	5.01	4.06
19	Isobutyraldehyde	61.5	4.08	2.82	2.41	—	2.26	2.14
20	Methanol	64.7	1.19	0.94	0.68	1.26	0.71	0.74
21	*n*-Hexane	69.0	2.70	2.29	2.05	2.68	2.27	2.03
22	*n*-Propyl bromide	70.8	6.09	4.95	4.03	5.25	3.90	3.63
23	Butyraldehyde	75.7	4.53	4.06	2.94	4.10	3.12	—
24	Carbon tetrachloride	76.7	8.23	5.46	4.56	6.73	4.79	4.17
25	Ethyl acetate	77.1	5.33	3.70	2.97	4.15	2.93	2.34
26	*n*-Butylamine	77.8	—	4.64	4.18	—	—	—
27	Ethanol	78.3	2.22	1.68	1.30	2.47	1.49	1.28
28	Acrylonitrile	79.0	4.23	3.34	2.68	3.44	2.49	2.00
29	Methyl ethyl ketone	79.6	5.89	4.44	3.47	4.36	3.21	2.37
30	Methyl propionate	79.7	6.27	4.32	3.53	4.94	3.13	2.71
31	Benzene	80.1	9.12	6.56	5.03	7.42	5.45	4.80
32	Methyl acrylate	80.5	6.24	4.38	3.09	5.00	3.42	3.06
33	Cyclohexane	81.4	5.42	4.58	3.95	5.27	4.02	4.00
34	Acetonitrile	81.6	2.88	2.73	2.03	2.51	1.95	1.80
35	Iso-propanol	82.0	2.96	2.16	1.35	3.46	2.13	1.92
36	*tert.*-Butanol	82.6	4.31	2.70	2.24	4.21	2.63	2.20
37	Cyclohexene	83.3	7.30	5.50	4.42	6.77	5.07	4.23
38	1,2-Dichloroethane	83.5	10.7	7.42	5.32	9.48	6.16	4.78
39	Thiophene	84.0	10.3	7.53	5.95	—	6.50	5.31
40	Trichloroethylene	87.2	13.1	8.70	6.29	12.0	7.28	5.68

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TO *n*-PENTANE (= 1) ON DIFFERENT STATIONARY PHASES

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Stationary phases:

A. Di-*n*-decyl phthalate	F. Tetraethylene glycol dimethyl ether
B. Diethylhexyl sebacate	G. Fluorene/picric acid
C. Silicone DC 200	K. Polyethylene glycol.

C			*F*	*G*	*K*			*Substance*	*No.*
50°	*100°*	*150°*	*50°*	*100°*	*50°*	*100°*	*150°*		
0.30	—	—	0.31	—	—	—	—	Isobutane	1
0.42	0.47	0.53	0.42	—	—	—	—	*n*-Butane	2
0.78	0.84	1.0	0.77	1.0	1.0	1.0	1.0	Isopentane	3
0.50	0.53	0.61	0.83	6.89	15.2	6.95	6.0	Methyl formate	4
0.90	1.07	1.28	1.66	4.55	15.9	8.82	6.0	Furan	5
1.00	1.03	1.07	1.25	2.22	3.93	3.22	2.67	Diethyl ether	6
1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	*n*-Pentane	7
1.20	1.37	1.32	3.13	6.34	37.1	19.5	15.0	Methylene chloride	8
1.40	1.50	1.36	2.51	5.44	8.85	5.76	5.00	Carbon disulphide	9
1.50	1.60	1.64	2.58	4.44	10.6	6.95	6.00	*n*-Propyl chloride	10
1.26	1.49	1.57	—	9.44	47.5	20.3	—	*n*-Propylamine	11
0.80	0.80	0.82	1.95	12.1	20.4	10.5	7.67	Propionaldehyde	12
0.78	0.74	0.71	2.11	15.0	31.3	13.9	12.7	Acrolein	13
1.30	1.23	1.03	2.10	11.3	23.6	11.7	10.7	Ethyl formate	14
0.82	0.96	0.97	2.10	17.0	24.2	13.7	11.7	Acetone	15
1.21	1.26	1.11	2.33	13.0	23.6	14.1	10.0	Methyl acetate	16
1.86	1.57	1.46	4.45	8.22	29.4	18.1	13.0	1.1-Dichloroethane	17
2.65	2.54	2.25	8.10	24.2	82.0	35.6	21.7	Chloroform	18
1.58	1.48	1.43	3.31	14.7	23.4	12.2	9.67	Isobutyraldehyde	19
0.35	0.34	0.36	1.21	8.8	62.2	23.4	15.0	Methanol	20
2.64	2.29	2.11	2.70	2.00	2.92	2.54	2.00	*n*-Hexane	21
2.83	2.77	2.50	5.95	11.5	25.8	15.4	9.33	*n*-Propyl bromide	22
2.16	1.97	1.64	5.15	18.8	35.6	19.3	14.3	Butyraldehyde	23
4.40	3.81	3.71	6.93	23.1	30.8	15.8	12.0	Carbon tetrachloride	24
2.80	2.36	2.10	4.91	20.3	38.4	19.3	13.7	Ethyl acetate	25
—	—	2.82	—	—	—	—	—	*n*-Butylamine	26
0.72	0.69	0.57	2.32	11.3	87.4	31.5	16.3	Ethanol	27
1.04	1.13	1.14	4.28	22.5	77.2	37.3	23.7	Acrylonitrile	28
2.20	2.07	1.82	5.57	29.7	47.0	22.7	17.3	Methyl ethyl ketone	29
3.04	2.40	2.11	5.79	22.9	44.9	22.6	15.7	Methyl propionate	30
4.48	3.66	3.07	8.62	20.4	51.7	26.1	18.7	Benzene	31
2.76	2.31	2.18	5.82	17.3	56.3	26.4	17.0	Methyl acrylate	32
4.64	3.74	3.07	7.20	4.9	9.30	6.60	5.67	Cyclohexane	33
0.89	0.93	1.04	3.30	28.6	102	42.7	27.0	Acetonitrile	34
0.97	1.03	1.00	2.61	10.8	80.1	28.8	15.3	Iso-propanol	35
1.41	1.28	1.25	4.20	9.56	65.4	23.8	12.3	*tert.*-Butanol	36
5.06	4.48	3.36	5.54	9.22	17.1	11.7	9.00	Cyclohexene	37
3.22	2.72	2.61	8.68	25.4	116	52.5	33.3	1,2-Dichloroethane	38
4.30	3.55	3.11	10.7	41.5	86.4	41.4	28.7	Thiophen	39
5.67	4.74	4.26	12.9	19.7	72.0	31.9	21.0	Trichloroethylene	40

(continued p. D12)

TABLE

No.	Substance	B.p. °C	A			B		
			50°	100°	150°	50°	100°	15
41	Isopropyl acetate	89.0	7.44	4.95	3.53	6.24	4.07	3.
42	Di-*n*-propyl ether	91.0	7.33	3.9	3.76	—	3.71	3.6
43	Methyl isobutyrate	92.3	8.90	6.16	4.38	—	4.84	3.
44	*n*-Propanol	97.2	6.79	3.91	2.88	5.38	3.75	2.
45	*n*-Heptane	98.4	7.15	5.04	4.03	7.22	4.93	3.9
46	Ethyl propionate	99.1	11.8	7.43	5.30	9.90	5.94	4.2
47	Iso-octane	99.3	6.34	4.98	4.09	6.40	4.61	3.8
48	*sec.*-Butanol	99.5	8.30	5.20	3.56	9.15	5.02	3.6
49	Ethyl acrylate	99.8	11.9	7.60	5.18	10.2	6.14	4.8
50	Water	100.0	1.55	1.45	1.25	—	2.24	1.9
51	Methyl methacrylate	100.0	13.2	8.44	5.97	—	6.72	5.0
52	Methylcyclohexane	100.3	10.0	7.44	5.82	—	6.58	5.5
53	Formic acid	100.7	—	5.72	3.70	—	8.68	—
54	1,4-Dioxane	100.8	14.4	9.35	6.80	—	7.61	6.1
55	*n*-Propyl acetate	101.6	12.8	7.81	5.30	—	6.39	4.9
56	2-Methylbutan-2-ol	102.0	11.4	6.82	4.71	—	6.38	4.6
57	Methyl *n*-butyrate	102.3	13.5	8.63	6.00	11.7	6.87	5.4
58	Diisobutylene	102.6	8.49	6.36	5.12	—	5.83	4.9
59	Diethyl ketone	102.7	—	9.01	6.09	10.5	6.68	5.2
60	Crotonaldehyde	104.0	—	8.43	5.64	—	6.00	4.6
61	Toluene	110.0	—	14.5	9.61	—	12.6	9.1
62	Ethyl isobutyrate	110.1	—	10.0	6.74	—	8.11	7.0
63	Pyridine	115.5	—	15.7	10.7	—	13.7	9.8
64	Isobutyl acetate	116.5	—	13.2	7.85	—	9.78	7.0
65	*n*-Butanol	117.0	20.0	9.11	5.74	—	8.72	5.5
66	Ethyl methacrylate	117.0	—	13.7	8.62	—	10.7	7.8
67	Crotyl alcohol	118.0	—	11.2	6.71	—	9.41	—
68	Acetic acid	118.1	—	9.36	5.59	—	10.95	—
69	Ethyl *n*-butyrate	120.0	—	14.5	9.24	—	12.2	8.1
70	Perchloroethylene	120.8	—	16.0	11.5	—	15.5	10.6
71	Paraldehyde	124.4	—	13.2	8.23	—	26.8	16.7
72	*n*-Octane	125.8	19.2	11.6	8.09	19.3	11.0	7.8
73	*n*-Butyl acetate	126.5	—	16.8	10.0	—	13.5	9.1
74	2-Methylbutan-4-ol	130.5	—	16.6	9.35	—	17.6	8.8
75	Monochlorobenzene	132.0	—	31.2	18.6	—	26.8	16.7
76	Ethylene glycol monoethyl ether	135.1	—	14.5	8.56	—	11.0	7.5
77	Ethylbenzene	136.2	—	29.9	17.1	—	25.1	15.9
78	*p*-Xylene	138.0	—	31.9	18.8	—	26.4	17.5
79	*prim. n*-Amyl alcohol	138.0	—	20.5	11.3	—	19.4	11.1
80	*m*-Xylene	139.0	—	31.4	18.6	—	26.5	17.5
81	Acetylacetone	139.0	—	22.9	14.2	—	17.5	11.7
82	Acetic anhydride	139.4	—	17.1	6.75	—	—	6.5
83	Propionic acid	140.0	—	26.0	—	—	25.1	12.2
84	Monochlorocyclohexane	142.5	—	35.0	21.6	—	—	18.7
85	*o*-Xylene	144.0	—	37.9	21.6	—	31.5	20.1
86	Styrene	146.0	—	41.7	24.4	—	36.9	21.5
87	Ethyl valerate	146.0	—	26.9	16.8	—	24.0	15.0
88	1,1,2,2-Tetrachloroethane	146.2	—	69.4	33.6	—	—	32.5
89	Dimethylformamide	152.4	—	—	16.2	—	—	—
90	Iso-butyric acid	154.4	—	—	17.4	—	—	17.7

ABLE 20 *(continued)*

	C			*F*	*G*	*K*			*Substance*	*No.*
150°	*50°*	*100°*	*150°*	*50°*	*100°*	*50°*	*100°*	*150°*		
3.57	4.18	3.04	2.64	7.31	22.9	42.5	20.9	13.3	Isopropyl acetate	41
3.60	5.57	4.33	3.46	7.11	8.12	14.9	8.99	7.66	Di-*n*-propyl ether	42
3.77	5.11	3.73	2.89	8.78	10.7	53.3	26.3	14.4	Methyl isobutyrate	43
2.52	1.99	1.51	1.36	6.20	20.8	180	59.3	29.0	*n*-Propanol	44
3.98	6.73	4.97	3.90	7.30	4.00	7.76	5.09	4.00	*n*-Heptane	45
4.26	6.46	4.60	3.33	11.6	33.8	69.2	31.7	20.3	Ethyl propionate	46
3.80	5.98	4.60	3.86	6.28	3.90	5.9	4.4	3.0	Iso-octane	47
3.60	2.44	2.23	2.07	8.72	21.3	138	51.9	22.0	*sec.*-Butanol	48
4.83	5.25	4.20	3.36	12.1	37.8	86.2	37.6	21.4	Ethyl acrylate	49
1.95	0.72	0.75	0.89	3.23	27.1	252	72.4	40.7	Water	50
5.02	6.09	4.70	3.46	13.6	39.6	93.5	41.8	23.0	Methyl methacrylate	51
5.57	7.98	6.21	4.65	10.0	7.44	14.2	9.32	7.67	Methylcyclohexane	52
—	—	—	—	—	—	—	688	252	Formic acid	53
6.12	5.49	4.70	3.42	14.0	104.9	157	71.3	38.0	1,4-Dioxane	54
4.94	6.45	4.63	3.50	12.5	34.1	71.8	34.9	20.2	*n*-Propyl acetate	55
4.60	3.41	3.06	2.57	11.9	22.2	—	56.0	22.7	2-Methylbutan-2-ol	56
5.43	6.92	5.20	3.75	14.4	39.0	84.7	39.4	22.0	Methyl *n*-butyrate	57
4.92	7.02	5.66	4.36	8.68	7.33	11.2	7.8	7.0	Diisobutylene	58
5.26	5.44	4.29	3.32	13.6	49.0	85.2	37.9	24.7	Diethyl ketone	59
4.60	3.39	3.22	2.82	12.8	—	—	58.4	35.7	Crotonaldehyde	60
9.10	10.8	7.75	5.58	24.1	48.9	115	49.0	31.3	Toluene	61
7.03	9.25	6.89	4.75	16.7	35.4	71.8	32.7	18.7	Ethyl isobutyrate	62
9.89	7.52	6.15	5.10	25.5	—	—	154	72.0	Pyridine	63
7.05	10.82	7.09	5.06	22.5	47.6	105	48.6	24.4	Isobutyl acetate	64
5.52	4.46	3.57	2.86	18.7	42.8	—	111	41.0	*n*-Butanol	65
7.80	11.8	7.92	5.42	25.4	55.5	—	—	27.7	Ethyl methacrylate	66
—	—	—	—	—	—	—	—	—	Crotyl alcohol	67
—	—	2.87	1.89	—	—	—	—	—	Acetic acid	68
8.18	13.2	8.60	5.90	26.3	53.0	—	48.5	30.3	Ethyl *n*-butyrate	69
10.6	16.2	10.7	7.35	—	21.2	109	44.9	27.0	Perchloroethylene	70
16.7	18.7	12.8	8.55	—	15.8	—	61.7	31.1	Paraldehyde	71
7.8	17.1	11.5	8.10	19.2	7.78	21.5	11.4	8.00	*n*-Octane	72
9.14	14.8	8.89	6.35	—	81.2	—	63.9	33.0	*n*-Butyl acetate	73
8.83	8.84	5.96	4.28	—	62.9	—	167	60.4	2-Methylbutan-4-ol	74
16.7	18.7	12.8	8.6	—	107.0	—	133	68.3	Monochlorobenzene	75
7.54	7.49	5.33	3.99	—	149.0	—	—	78.0	Ethylene glycol monoethyl ether	76
15.9	—	14.6	9.40	—	79.4	—	83.6	45.7	Ethylbenzene	77
17.5	26.0	15.6	9.96	—	105.0	—	88.2	47.3	*p*-Xylene	78
11.1	10.8	7.15	5.08	—	85.8	—	200,0	76.0	*prim. n*-Amyl alcohol	79
17.5	—	14.3	9.71	—	109.0	—	91.0	48.0	*m*-Xylene	80
11.7	11.9	8.10	6.11	—	24.4	—	159.0	—	Acetylacetone	81
6.50	—	4.79	—	—	76.1	—	—	184.0	Acetic anhydride	82
12.2	—	—	—	—	—	—	—	287.0	Propionic acid	83
18.7	—	16.5	11.4	—	—	—	91.8	56.3	Monochlorocyclohexane	84
20.1	—	19.2	11.3	—	126.5	—	118.7	60.4	*o*-Xylene	85
21.5	—	19.3	10.8	—	163.0	—	167.0	79.6	Styrene	86
15.0	—	15.3	10.0	—	—	—	86.0	47.3	Ethyl valerate	87
32.5	—	18.3	11.7	—	210.0	—	—	191.0	1,1,2,2-Tetrachloroethane	88
—	12.1	7.53	3.86	—	—	—	—	195.0	Dimethylformamide	89
17.7	—	13.5	6.54	—	—	—	—	—	Isobutyric acid	90

(continued p. D14)

TABLE 20

No.	*Substance*	*B.p. °C*	*A*		*B*	
			100°	*150°*	*100°*	*150°*
91	Cyclohexanone	155.0	54.5	27.6	47.8	24.0
92	Bromobenzene	155.6	—	33.6	—	29.3
93	Camphene	156.0	—	24.6	—	23.1
94	*prim. n*-Hexanol	157.2	—	21.1	—	—
95	Cyclohexanol	160.5	51.3	29.2	35.2	21.0
96	Furfural	161.6	—	22.3	—	18.6
97	*n*-Butyric acid	162.5	—	—	—	—
98	Ethyl caproate	166.0	—	30.7	—	28.2
99	*prim. n*-Heptyl alcohol	176.3	—	41.5	—	34.7
100	Dipentene	177.6	—	32.4	—	—
101	Benzaldehyde	178.0	—	—	—	—
102	Diethylformamide	178.0	—	36.1	—	—
103	*o*-Dichlorobenzene	179.0	—	64.8	—	—
104	Aniline	184.0	—	64.5	—	—
105	*n*-Valeric acid	187.0	—	—	—	—
106	Ethyl oenanthate	187.0	—	—	—	—
107	1,2-Propylene glycol	189.0	—	—	—	—
108	*o*-Cresol	191.0	—	139.0	—	—
109	Methyl caprylate	192.9	—	65.4	—	—
110	Acetonylacetone	194.0	—	—	—	—
111	*prim. n*-Octyl alcohol	194.5	—	—	—	—
112	Ethylene glycol	197.4	—	—	—	—
113	Methyl benzoate	199.5	—	—	—	—
114	*m*-Cresol	202.0	—	—	—	—
115	*p*-Cresol	202.0	—	—	—	—
116	Ethyl caprylate	208.0	—	—	—	—
117	Formamide	210.7	—	—	—	—
118	Nitrobenzene	210.9	—	—	—	—
119	2,4-Dimethylphenol	211.5	—	—	—	—
120	1,3-Propylene glycol	214.0	—	—	—	—

(continued)

C		K		Substance	No.
100°	*150°*	*100°*	*150°*		
16.3	11.6	—	158	Cyclohexanone	91
21.8	14.3	—	124	Bromobenzene	92
26.4	16.5	52.6	37	Camphene	93
14.6	10.5	—	118	*prim. n*-Hexanol	94
17.1	12.4	—	131	Cyclohexanol	95
13.3	9.04	—	214	Furfural	96
—	7.36	—	—	*n*-Butyric acid	97
—	17.7	—	70.3	Ethyl caproate	98
—	17.5	—	189	*prim. n*-Heptyl alcohol	99
—	21.4	—	51	Dipentene	100
—	14.8	—	282	Benzaldehyde	101
—	20.6	—	290	Diethylformamide	102
—	23.9	—	235	*o*-Dichlorobenzene	103
—	18.2	—	672	Aniline	104
—	15.9	—	—	*n*-Valeric acid	105
—	29.5	—	108	Ethyl oenanthate	106
—	9.6	—	1533	1,2-Propylene glycol	107
—	16.1	—	1450	*o*-Cresol	108
—	35.1	—	139	Methyl caprylate	109
—	14.8	—	286	Acetonylacetone	110
—	32.9	—	310	*prim. n*-Octyl alcohol	111
—	25.6	—	682	Ethylene glycol	112
—	—	—	397	Methyl benzoate	113
—	—	—	2110	*m*-Cresol	114
—	26.7	—	2030	*p*-Cresol	115
—	—	—	174	Ethyl caprylate	116
—	—	—	1650	Formamide	117
—	—	—	722	Nitrobenzene	118
—	—	—	2440	2,4-Dimethylphenol	119
—	—	—	1530	1,3-Propylene glycol	120

TABLE 21

R_F VALUES OF ALKALI IONS

(H. J. ARNIKAR, *Nature*, 182 (1958) 1230)

Paper: acid washed asbestos-paper.
Solvent: 0.1 *N* aqueous HCl.

Ion	R_F
Li^+	0.99
Na^+	0.86
K^+	0.77
Rb^+	0.72
Cs^+	0.45

TABLE 22

R_F VALUES OF SULPHURIC ACID DERIVATIVES

(H. A. LEHMANN AND G. KEMPE, *Chem. Tech.* (*Berlin*), 9 (1957) 719)

Solvent: dioxane–water–1% ammonia (75:24:1).
Paper: Schleicher and Schüll 2043 a.

Substance (as ammonium salt)	R_F
Sulphate	0.05
Imidodisulphate	0.18
Amidosulphate	0.36
Imidodisulphamide	0.52
Sulphamide	0.84

TABLE 23

R_F VALUES OF VASOPRESSINS AND OXYTOCINS

(H. HELLER AND K. LEDERIS, *Nature*, 182 (1958) 1231)

Solvent: *n*-butanol–acetic acid–water (5:1:4).

Peptide	R_F
Arginine-vasopressin	0.19
Lysine-vasopressin	0.26
Oxytocin (synthetic)	0.37–0.40
Valine-oxytocin	0.33
Phenylalanine-oxytocin	0.37–0.39

TABLE 24

R_F VALUES OF CONSTITUENTS OF HAIR DYES

(C. I. TURI, *Rend. ist. super. sanità*, 21 (1958) 748)

Substance	*Butanol* 4 *Acetic acid* 1 *Water* 5	*Butanol* 4 *Ethanol* 1 *Water* 5
Resorcinol	0.90	0.92
Pyrocatechol	0.90	0.92
Hydroquinone	0.90	0.91
Pyrogallol	0.70	0.74
o-Aminophenol	0.72	0.82
m-Aminophenol	0.70	0.74
p-Aminophenol	0.57	0.74
2,4-Diaminophenol	0.42	0.38
Methyl-*p*-aminophenol	0.69	0.58
Glycine	0.50	0.51
4-Nitro-2-aminophenol	0.93	0.88
Picramic acid	0.85	0.52
o-Phenylenediamine	0.66	0.74
m-Phenylenediamine	0.53	0.58
p-Phenylenediamine	0.48	0.54
Nitro-*o*-phenylenediamine	0.78	0.73
Nitro-*p*-phenylenediamine	0.67	0.73
Acetyl-*p*-phenylenediamine	0.63	0.73
1,2,5-Tolylenediamine	0.52	0.55
1,2,4-Tolylenediamine	0.59	0.70
p-Aminodiphenylamine	0.82	0.89
Diaminodiphenylamine	0.46	0.66
p-Phenylenediamine-sulphonic acid	0.19	0.13
p-Aminodiphenylamine-sulphonic acid	0.56	0.44
Diaminodiphenylamine-sulphonic acid	0.23	0.24
2-Aminophenol-4-sulphonic acid	0.20	0.17

TABLE 25

R_F VALUES OF NITROBENZOIC ACIDS

(V. ETTEL, J. POSPISIL AND Z. DEYL, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 234)

Paper: Whatman No. 1.

Acid	*Solvent*	
	Ethanol 95 *Ammonia* 5	*Ethyl acetate* 95 *Ammonia* 5
o-Nitrobenzoic	1.0	
m-Nitrobenzoic	0.88	
p-Nitrobenzoic	0.75	0.09
2,4-Dinitrobenzoic	0.85	
2,6-Dinitrobenzoic	1.0	
3,4-Dinitrobenzoic	1.0	
3,5-Dinitrobenzoic	0.72	0.17
2,3,5-Trinitrobenzoic	0.83	
2,3,6-Trinitrobenzoic	1.0	
2,4,5-Trinitrobenzoic	0.70	
2,4,6-Trinitrobenzoic	0.63	0.07

TABLE 26

R_F VALUES OF NITROBENZOIC ACIDS

(V. ETTEL, J. POSPISIL AND Z. DEYL, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 234)

Paper: Whatman No. 4 impregnated with kerosene (5% in benzene).
Solvent: ethyl acetate–ammonia (95:5).

Substance	R_F
2,4-Dinitrobenzoic acid	0.34
2,6-Dinitrobenzoic acid	0.60
3,4-Dinitrobenzoic acid	0.60
3,5-Dinitrobenzoic acid	0.24
2,3,5-Trinitrobenzoic acid	0.32
2,3,6-Trinitrobenzoic acid	0.48
2,4,5-Trinitrobenzoic acid	0.19
2,4,6-Trinitrobenzoic acid	0.16

TABLE 27

R_F VALUES OF NITROBENZOIC ACIDS

(V. ETTEL, J. POSPISIL AND Z. DEYL, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 234)

Paper: Whatman No. 1 impregnated with formamide (5% in ethanol).

Substance	*Solvent*	
	Benzene	*Ethyl acetate*
o-Nitrobenzoic acid	1.0	
m-Nitrobenzoic acid	0.76	
p-Nitrobenzoic acid	0.67	0.10
2,4-Dinitrobenzoic acid	0.59	
2,6-Dinitrobenzoic acid	0.84	
3,4-Dinitrobenzoic acid	0.83	
3,5-Dinitrobenzoic acid	0.48	0.19
2,4,6-Trinitrobenzoic acid		0.12

TABLE 28

R_F VALUES OF NITROPHENOLS

(V. ETTEL, J. POSPISIL AND Z. DEYL, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 234)

Paper: Whatman No. 4 impregnated with formamide (5% in ethanol).

Substance	*Solvent*		
	Benzene	*Chloroform*	*Ethyl acetate*
o-Nitrophenol	1.0	0.66	0.70
m-Nitrophenol	0.89	0.75	0.74
p-Nitrophenol	0.84	0.66	0.70
2,4-Dinitrophenol	0.77	0.63	0.55
2,6-Dinitrophenol	0.55	0.52	0.60

TABLE 29

R_F VALUES OF NITRO DERIVATIVES OF BENZENE AND TOLUENE

(V. ETTEL, J. POSPISIL AND Z. DEYL, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 234)

Paper: Whatman No. 1 impregnated with formamide (5% in ethanol).

Substance	*Developed with*	
	n-Hexane	*Cyclohexane*
o-Nitrotoluene	0.96	
p-Nitrotoluene	0.90	
2,4-Dinitrotoluene	0.84	0.70
2,6-Dinitrotoluene	0.80	0.66
3,4-Dinitrotoluene		0.91
3,5-Dinitrotoluene	0.86	0.72
1,2,3-Trinitrobenzene		1.00
1,2,4-Trinitrobenzene	0.74	0.60
1,3,5-Trinitrobenzene	0.34	0.20
2,3,5-Trinitrotoluene	0.69	0.68
2,3,6-Trinitrotoluene	0.66	0.64
2,4,5-Trinitrotoluene	0.72	0.70
2,4,6-Trinitrotoluene	0.32	0.31

TABLE 30

R_F VALUES OF SOME NITRO COMPOUNDS

(T. KIKAL AND J. N. SMITH, *Biochem. J.*, 71 (1959) 48)

Paper: Whatman No. 1 or No. 4.

Solvents: A. Benzene–butanol–conc. NH_4OH (2:5:2).
B. Benzene–acetic acid–water (1:1:2).
C. Butanol–acetic acid–water (4:1:5).
D. Butanol saturated with water.
E. Methyl ethyl ketone saturated with water.
F. Methyl ethyl ketone saturated with 2 *N* ammonia.
G. Propanol–conc. NH_4OH (7:3).

Substance	*Solvent*						
	A	*B*	*C*	*D*	*E*	*F*	*G*
6-Amino-4-nitro-*o*-cresol	0.25	0.33	0.91	0.86	0.32	0.47	0.63
6-Amino-4-nitro-*o*-cresyl sulphate	0.49	0	0.57	0.51	0.80	—	0.75
6-Amino-4-nitro-*o*-cresyl glucoside	0.70	0	0.77	0.60	0.80	—	0.75
6-Acetamido-4-nitro-*o*-cresol	0.53	0.78	0.91	0.82	0.92	0.57	0.75
6-Acetamido-4-nitro-*o*-cresyl sulphate	0.65	0	0.56	0.64	0.83	—	0.84
6-Acetamido-4-nitro-*o*-cresyl glucoside	0.70	0	0.82	0.69	0.83	—	0.75
3-Amino-5-nitrosalicylic acid	0.06	0.07	0.69	0.26	0.74	0.67	—
3-Acetamido-5-nitrosalicylic acid	0.06	0.01	0.62	0.33	0.56	0.55	—
4,6-Dinitro-*o*-cresol	0.75	0.95	0.95	—	—	0.85	—
2-Amino-4-nitrophenol	0.13	—	—	—	0.95	0.33	—
2-Amino-4-nitrophenyl sulphate	0.25	—	—	—	0.37	0.51	—

TABLE 31

R_F VALUES OF AROMATIC NITROCARBOXYLIC ACIDS

(J. Franc, *Collection Czechoslov. Chem. Communs.*, 23 (1958) 2018; See also *This journal*, 1 (1958) xli)

Paper: Whatman No. 1.
Temperature: 18 ± 1°.

Acid	*Butanol* 5 *Pyridine* 3 *Water* 3	*Isoamyl alcohol* 1 *Ethanol* 1 *Pyridine* 1 *Water* 1
3-Nitro-*o*-toluic	0.77	
5-Nitro-*o*-toluic	0.70	
3,5-Dinitro-*o*-toluic	0.73	0.73
4,5-Dinitro-*o*-toluic	0.25	
3,5,6-Trinitro-*o*-toluic	0.29	0.42
2-Nitro-*m*-toluic	0.67	0.67
6-Nitro-*m*-toluic	0.63	0.62
2,5-Dinitro-*m*-toluic	0.75	0.72
2,6-Dinitro-*m*-toluic	0.26	
4,6-Dinitro-*m*-toluic	0.42	0.50
2,4,5-Trinitro-*m*-toluic	0.39	
2,5,6-Trinitro-*m*-toluic	0.16	0.34
3-Nitro-*p*-toluic	0.73	0.74
2,3-Dinitro-*p*-toluic	0.15	0.31
2,5-Dinitro-*p*-toluic	0.68	0.70
3,5-Dinitro-*p*-toluic	0.72	0.73
2,3,6-Trinitro-*p*-toluic	0.19	0.28
5-Nitrohemimellitic	0.21	
5-Nitrotrimellitic	0.14	
Sulphuric	0.06	0.07
Nitric	0.50	0.53

TABLE 32

R_F VALUES OF SOME AROMATIC CARBOXYLIC ACIDS

(J. Franc, *Collection Czechoslov. Chem. Communs.*, 23 (1958) 2018)

Solvent: butanol–pyridine–water (3:1:1).
Paper: Whatman No. 4.
Temperature: 19°.

Acid	R_F *value*
o-Toluic	0.80
m-Toluic	0.78
p-Toluic	0.76
Hemimellitic	0.055
Trimellitic	0.065
Trimesic	0.055

TABLE 33

R_F VALUES OF AMINOANTHRAQUINONES

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 250)

Paper: Whatman No. 4.
Temperature: 21 ± 1°.
Impregnation of paper: 1. Formamide.
2. Acetamide.
3. Urea.
4. Dimethylformamide.

Anthraquinone derivative	*R_F values*			
	1	*2*	*3*	*4*
Solvent: chloroform				
1-Amino	0.985	0.95	1.00	0.74
2-Amino	0.92	0.86	0.96	0.49
1,2-Diamino	0.78	0.75	0.84	0.35
1,4-Diamino	0.89	0.81	0.925	0.35
1,5-Diamino	0.94	0.89	0.96	0.46
2,6-Diamino	0.25	0.27	0.17	0.07
1-Amino-4-hydroxy	0.97	0.965		0.52
Solvent: cyclohexane–chloroform (1:1)				
1-Amino	0.945	0.895	0.995	
2-Amino	0.71	0.68	0.94	
1,2-Diamino	0.30	0.27	0.78	
1,4-Diamino	0.55	—	0.90	
1,5-Diamino	0.78	0.71	0.95	
2,6-Diamino	0.02	0.02	0.04	
1-Amino-4-hydroxy	0.91	0.91	0.985	

TABLE 34

R_F VALUES OF AMINOANTHRAQUINONES

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 250)

Paper: Whatman No. 4.
Solvent: cyclohexane–pyridine (25:1).
Temperature: 20 ± 1°.
Impregnation of paper: 1. Acetamide.
2. Formamide.
3. Dimethylformamide.

Anthraquinone derivative	*R_F values*		
	1	*2*	*3*
1-Amino	0.39	0.42	0.14
2-Amino	0.02	0.03	0.02
1,2-Diamino	0.01	0.01	0.01
1,4-Diamino	0.01	0.02	0.01
1,5-Diamino	0.06	0.08	0.01
2,6-Diamino	0.03	0.00	0.00
1-Amino-4-hydroxy	0.32	0.39	0.14

TABLE 35

R_F VALUES OF HYDROXYANTHRAQUINONES

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 250)

Paper: Whatman No. 4.
Solvent: cyclohexane–pyridine (25:1).
Temperature: 19 ± 1°.
Impregnation of paper: 1. Acetamide.
2. Formamide.
3. Dimethylformamide.
4. Amidosulphonic acid.
5. Water (elongated spots).
6. Urea.
7. Phosphoric acid.
8. Monoethanolamine.
9. Petrol (Solvent: ethanol–water–formamide 8:2:1).

Anthraquinone derivative	*R_F values*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1-Hydroxy	0.87	0.78	0.72	0.93	0.89	0.51	1.00	0.96	0.64
2-Hydroxy	0.20	0.13	0.06	0.00	0.19	0.945	0.02	0.03	0.98
1,2-Dihydroxy	0.22	0.05	0.07	0.00	0.17	0.28	0.12	0.00	
1,4-Dihydroxy	0.88	0.76	0.67	0.91	0.88	0.89	0.92	0.95	0.53
1,5-Dihydroxy	0.88	0.76	0.67		0.87	0.89	0.92	0.94	0.53
1,6-Dihydroxy	0.31	0.16	0.09	0.04	0.29	0.55	0.03	0.03	0.96
1,8-Dihydroxy	0.81	0.72	0.61	0.91	0.82	0.91	0.94	0.78	0.56
2,6-Dihydroxy	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.92
2,7-Dihydroxy	0.00	0.00	0.00	0.00	0.00			0.00	

TABLE 36

R_F VALUES OF AROMATIC AMINO COMPOUNDS

(V. ETTEL, J. POSPISIL AND Z. DEYL, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 234)

Paper: Whatman No. 1.
Solvent: ethanol–ammonia (95:5).

Substance	R_F	*Substance*	R_F
o-Aminotoluene	0.84	*p*-Aminophenol	0.60
p-Aminotoluene	0.89	2,4-Diaminophenol	0.16
2,4-Diaminotoluene	0.75	2,6-Diaminophenol	0.60
2,6-Diaminotoluene	0.66	2,4,6-Triaminophenol	0.60
3,4-Diaminotoluene	0.90	*o*-Aminobenzoic acid	0.32
3,5-Diaminotoluene	0.72	*m*-Aminobenzoic acid	0.40
1,2,3-Triaminobenzene	1.0	*p*-Aminobenzoic acid	0.46
1,2,4-Triaminobenzene	0.79	2,4-Diaminobenzoic acid	0.21
1,3,5-Triaminobenzene	0.63	2,6-Diaminobenzoic acid	0.09
2,3,5-Triaminotoluene	0.77	3,4-Diaminobenzoic acid	0.36
2,3,6-Triaminotoluene	0.74	3,5-Diaminobenzoic acid	0.28
2,4,5-Triaminotoluene	0.74	2,3,5-Triaminobenzoic acid	0.80
2,4,6-Triaminotoluene	0.62	2,3,6-Triaminobenzoic acid	0.66
o-Aminophenol	0.64	2,4,5-Triaminobenzoic acid	0.86
m-Aminophenol	0.60	2,4,6-Triaminobenzoic acid	0.68

TABLE 37

R_F VALUES OF KHELLIN, VISNAGIN AND KHELLOL GLUCOSIDE

(A. A. A. RAHMAN AND A. A. KASSIM, *Proc. Pharm. Soc. Egypt*, 37 (1955) 75)

Solvent	*R_F of khellin or visnagin*	*R_F of khellol glucoside*
Butanol–10% acetic acid sat. with water	0.87	0.41
Butanol–30% acetic acid sat. with water	0.92	0.71
Butanol–2% conc. HCl sat. with water	0.90	0.40
Butanol–10% conc. HCl sat. with water	0.97	0.71
Butanol–2% conc. HNO_3 sat. with water	0.89	0.31
Butanol–2% conc. H_2SO_4 sat. with water	0.88	0.40

TABLE 38

R_F VALUES OF KHELLIN AND VISNAGIN

(A. A. A. RAHMAN AND A. A. KASSIM, *Proc. Pharm. Soc. Egypt*, 38 (1956) 79)

Solvent	*R_F of khellin*	*R_F of visnagin*
1% aqueous HCl	0.25	0.13
2% aqueous HCl	0.19	0.11
1% aqueous HNO_3	0.23	0.11
2% aqueous HNO_3	0.26	0.14
1% aqueous H_2SO_4	0.27	0.16
2% aqueous H_2SO_4	0.24	0.15

TABLE 39

R_F VALUES OF ALKALOIDS

(A. A. A. RAHMAN, *Arch. Pharm.*, 290 (1957) 321)

Alkaloid	*Butanol 100 Acetic acid 30 Water to saturation*	*Butanol 100 Conc. HCl 12.5 Water to saturation*
Morphine hydrochloride	0.72	0.68
Codeine phosphate	0.82	0.78
Dionine	0.84	0.68
Heroine	0.84	0.88
Narcotine hydrochloride	0.90	0.92
Papaverine hydrochloride	0.88	0.92
Thebaine hydrochloride	0.89	0.94
Dihydrocodeinone ditartrate	0.78	0.88
Dihydromorphinone hydrochloride	0.83	0.79
Hydroxydihydrocodeinone hydrochloride	0.77	0.74

TABLE 40

ELECTROMIGRATION OF SOME NITRO COMPOUNDS

(T. KIKAL AND J. N. SMITH, *Biochem. J.*, 71 (1959) 48)

Paper: Whatman No. 1.
Time: 2 h.
Voltage: 12 V/cm.
Buffers: 1. 0.02 *N* HCl.
2. pH 3.9 Britten and Robinson buffer.
3. pH 6.0 Britten and Robinson buffer.
4. pH 8.3 Britten and Robinson buffer.
5. 0.02 *N* NaOH.

Substance	*Buffer*				
	1	*2*	*3*	*4*	*5*
6-Amino-4-nitro-*o*-cresol	− 3.0	− 0.2	+ 0.1	+ 0.2	+ 5.0
6-Amino-4-nitro-*o*-cresyl sulphate	− 0.5	+ 4.3	+ 4.3	+ 4.7	+ 5.2
6-Amino-4-nitro-*o*-cresyl glucoside	− 1.5	− 0.2	− 0.7	− 0.1	− 1.0
6-Acetamido-4-nitro-*o*-cresol	− 0.5	0.0	+ 0.1	0.0	+ 4.9
6-Acetamido-4-nitro-*o*-cresyl sulphate	+ 2.6	+ 4.6	+ 4.0	+ 4.2	+ 5.0
6-Acetamido-4-nitro-*o*-cresyl glucoside	− 0.5	0.0	0.0	0.0	− 1.0
4,6-Dinitro-*o*-cresol	− 0.5	—	—	—	+ 5.0

TABLE 41

PAPER ELECTROPHORESIS OF SOME ACIDS

(O. THEANDER, *Svensk Kem. Tidskr.*, 70 (1958) 393)

Mobilities in cm/h with 35 V/cm on Whatman No. 1 paper.
Buffer concentration 0.05 *M*.

	pH2 (sulphate)	*pH4 (acetate)*	*pH7 (phosphate)*
Gluconic acid	< 1.5	0, 7.7	14.3
Galactonic acid	< 1.5	0, 8.4	14.5
Arabonic acid	< 1.5	0, 8.6	15.3
Xylonic acid	< 1.5	0, 9.1	15.8
Erythronic acid		10.5	18.1
Glyoxylic acid		15.4	24.7
2-Ketogluconic acid	3.2	10.7	14.9
5-Ketogluconic acid	1.5	9.3	14.6
α-Saccharinic acid	< 1.5	0, 7.7	13.7
α-Isosaccharinic acid	< 1.5	0, 8.1	13.9
Glucuronic acid	1.9	0, 9.3	14.0
Galacturonic acid	< 1.5	7.9	13.7
2-O-(4-O-Methyl-α-glucuronosyl)-xylose	< 1.5	0, 6.9	9.9
4-O-Methyl-glucuronic acid	< 1.5	0, 9.2	13.8
Digalacturonic acid	< 1.5 1.7	8.1, 10.2	13.2, 16.5
Glucaric acid	< 1.5 1.5 2.6	9.4, 10.8, 12.6	15.6, 24.6
o-Hydroxybenzoic acid		12.5	17.6
m-Hydroxybenzoic acid		8.4	16.2
p-Hydroxybenzoic acid		3.1	15.6

TABLE 42

R_F VALUES OF CATIONS IN SOLVENTS CONTAINING HF

(A. GRAND-CLEMENT, Z. JAKOVAC, M. LEDERER AND E. PLUCHET, *Microchemistry Symposium*, BIRMINGHAM, AUGUST 1958)

Ion	*R_F values*		
	Butanol 100 *HCl conc. 50* *HF conc. 2* *Water 48* *(one phase)*	*Butanol 100* *H_2SO_4 conc. 9.5* *Water 85.5* *HF conc. 5* *(two phases)*	*Acetone 90* *HCl conc. 5* *HF conc. 1* *Water 4* *(one phase)*
Ag^+	0.17–0.77 comet	0.08	0.46–0.59 comet
Hg^+	1.00	0.01	0.92
Pb^{+2}	0.44		0.48
Cu^{+2}	0.53	0.0	0.56
Cd^{+2}	0.92	0.07 slight comet	0.77
Bi^{+3}	0.75	0.0	0.85
Hg^{+2}	1.00	0.01	1.00
As^{+3}	0.92		0.65
AsO_4^{-3}	0.86	0.44	0.59
Sb^{+3}	0.88	0.22	0.82
Sn^{+2}	0.91		0.82
Sn^{+4}			
Al^{+3}	0.42	0.0	0.02
Cr^{+3}	0.38		0.27
Fe^{+3}	0.79	0.0	0.86
Zn^{+2}	0.93	0.0–0.24	0.90
Mn^{+2}	0.48	0.19	0.25
Co^{+2}	0.45	0.0	0.45
Ni^{+2}	0.52	0.0	0.03
Ca^{+2}	0.4 comet		0.0
Sr^{+2}	0.3		0.0
Ba^{+2}	0.22		0.0
Mg^{+2}	0.47		0.03
Li^{+2}	0.46		0.04
K^+	0.41		0.05
Rb^+	0.43		0.05
Cs^+	0.46		0.03
Th^{+4}	0.0		0.0
UO_2^{+2}	0.46	0.08	0.64
Tl^+	0.41 comet	0.04	1.00
Tl^{+3}	0.41–1.00		1.00
MoO_4^{-2}	0.73	0.41	0.92 comet
Be^{+2}	0.60		0.22
In^{+3}	0.57	0.05	0.82
Ga^{+3}	1.00	0.05	0.68
La^{+3}	0.0	0.0	0.0
Ce^{+3}	0.0	0.0	0.0
Y^{+3}	0.0		0.0
Sc^{+3}	0.0		0.0
Ti^{+4}	0.64	0.29	0.64
Zr^{+4}	0.52 comet	0.35	0.48 comet
Hf^{+4}	0.53 comet	0.35	0.46 comet
Nb^{+5}	0.88	0.51	0.94
Ta^{+5}	1.00	0.57	1.00
$^{233}Pa^{+5}$	0.45	0.08	0.62
V^{+5}	0.47	0.04	0.64
W	comet	0.38	comet
SeO_3^{-2}	0.72	0.57	1.00
TeO_3^{-2}	0.86	0.15	0.16
Pd^{+2}	0.83 comet		0.90 comet
Pt^{+5}	0.78–1.00		0.93
Ge^{+4}	0.59		0.29
SO_4^{-2}	0.74		0.48

TABLE 43

PAPER ELECTROPHORESIS OF INORGANIC CATIONS

(G. CETINI, *Atti accad. sci. Torino*, 91 (1956–57))

Electrolytes:

1. 25 ml 4 *N* lactic acid and 25 ml 2 *N* Na_2CO_3 made up to 1 l.
2. 40.02 ml of 0.2 *N* HCl and 250 ml of 0.2 *M* potassium hydrogen phthalate made up to 1 l.
3. 468 ml of 0.1 *M* sodium citrate and 532 ml of 0.1 *N* HCl.

Mobilities are relative to the movement of $Sr^{++} = 1$.

Cation	*Electrolyte*		
	1	*2*	*3*
Sr	1	1	1
Ba	0.98	0.96	0.98
Ca	0.93	0	0
Mn	0.73	0.65	0.81
Cd	0.55	0.46	0.62
Zn	0.42	0.18	0.50
Ni	0.34	0.28	0.62
Co	0.58	0.36	0.8
Pb	0.40	0	0.73
Cu	0.18	—0.40	0.47
Al	0.10	0	0
Bi	0.05	0	0
Hg(II)	0	0	0
As(III)	0		
Au(III)	0		
Sb(III)	—0.1		
Pd	—0.13		
Pt(II)	—0.21		
MoO_4^{-2}	—0.24		
WO_4^{-2}	—0.28		
Fe(III)	—0.32	—0.73	—0.22

TABLE 44

R_F VALUES OF V, Mo AND W IN SEVERAL SOLVENTS

(TZOU SHIH-FU AND LIANG SHU-CHUAN, *Sci. Sinica* (*Peking*), 8 (1959) 196)

Solvents	*R_F values*		
	V	*Mo*	*W*
Butanol–H_2O_2–*N* HNO_3 (20 : 1 : 5)	0.09	0.33	0.05
Butanol–H_2O_2–0.1 *N* HNO_3 (20 : 1 : 5)	0.09	0.33	0.05
Butanol–H_2O_2– *N* HNO_3 (20 : 5 : 1)	0.18	0.34	0.10
Butanol–H_2O_2–dioxane–*N* HNO_3(25 : 5 : 5 : 5)	0.35	0.62	0.32
Butanol–H_2O_2–benzoyl acetone–*N* HNO_3 (25 : 5 : 0.5 : 5)	0.24	0.54	0.24
Butanol 20 ml, $(NH_4)_2S_2O_8$ 1 g, 0.05 *N* HNO_3 5 ml	0.09	0.26	0.01
Butanol 25 ml, $(NH_4)_2S_2O_8$ 1 g, *N* HNO_3 5 ml	—0.03	0.35	0.01

TABLE 45

R_F VALUES OF COBALT IN MIXTURES OF TRI-*n*-BUTYL PHOSPHATE, METHANOL AND HCl

(G. R. Weidmann, *Can. J. Chem.*, 37 (1959) 830)

Paper: Schleicher and Schüll No. 4023b (Whatman No. 1).
Temperature: 22°.
Solvent: tri-*n*-butyl phosphate (TBP) was adjusted with HCl and the organic phase then mixed with methanol in the ratio 2:1 or 1:1.

Concn. of HCl used for adjusting TBP	*R_F values*		
	TBP–methanol (1:1)	*TBP–methanol (2:1)*	*Pure TBP*
5.5 *N*	—	0.16	—
6.5 *N*	—	0.23	—
7.5 *N*	—	0.28	—
8.5 *N*	—	0.34	—
9.0 *N*	0.36	0.37 0.38	0.7
9.5 *N*	—	0.43	—
10 *N*	0.46	0.46	0.85
10.5 *N*	—	0.48	—
11 *N*	—	0.52	—
11.5 *N*	—	0.59	—
12 *N*	—	0.66	—
12.5 *N*	—	0.74	—

TABLE 46

SEPARATION OF THIOUREA AND THIOURACIL

(M. Vietti-Michelina, *Z. anal. Chem.*, 167 (1959) 352)

Solvent A: *n*-butanol–acetic acid–water (5:1:4).
Solvent B: *n*-butanol–conc. ammonia (4:1).
Solvent C: *n*-butanol–water.

	R_F values	
	Thiourea	*Thiouracil*
Solvent A		
ascending	0.44	0.58
descending	0.45	0.58
Solvent B		
ascending	0.32	0.13
descending	0.32	0.08
Solvent C		
ascending	0.36	0.50
descending	0.47	0.62

TABLE 47

RETENTION VOLUMES OF SOME ORGANIC COMPOUNDS

(H. KELKER, *Angew. Chem.*, 71 (1959) 218)

Compound	*β,β'-Hydroxydipropionitrile V_i°, Benzene*	*Hexaethylene glycol dimethyl ether V_i°, Benzene*
n-Heptane	0.07_5	0.29
n-Octane	0.13	0.62
n-Decane	0.47	
Cyclohexane	0.11	0.30
Methylcyclohexane	0.14	0.46
Benzene	1.00	1.00
Methyl chloride	0.08_5	0.06_5
Methylene chloride	0.64	0.75
Chloroform	0.91	1.6_3
Carbon tetrachloride	0.36	0.70
Ethyl chloride	0.14	0.14
Vinyl chloride	0.07	0.08
1,1-Dichloroethane	0.58	0.68
1,2-Dichloroethane	2.1_5	2.0_6
1,1-Dichloroethylene	0.16	0.24
trans-1,2-Dichloroethylene	0.35	0.54
cis-1,2-Dichloroethylene	0.87	$1,2_4$
1,1,1-Trichloroethane	0.49	0.74
1,1,2-Trichloroethane	5.8	>4
Trichloroethylene	0.77	1.4_3
Perchloroethylene	0.98	1.9_3
Ethylene oxide	0.37	0.16
Propylene oxide	0.48	0.24
Isobutylene oxide	0.52	0.31
cis-2,3-Butylene oxide	0.78	0.46
trans-2,3-Butylene oxide	0.57	0.35
1,2-Butylene oxide	0.78	0.52
Acetaldehyde	0.43	0.16
Propionaldehyde	0.72	0.34
Isobutyraldehyde	0.78	0.46
n-Butyraldehyde	1.1_9	0.69
Acrolein	0.96	0.46
Crotonaldehyde	3.9_6	1.8_8
Acetone	1.1_0	0.41
Methyl ethyl ketone	1.6_7	0.79
Diethyl ketone	2.4_0	1.4_6
Methyl isopropyl ketone	1.8_9	1.0_8
Methyl *n*-propyl ketone	2.4_7	1.4_6
Formaldehyde dimethyl acetal	0.40	0.25
Formaldehyde diethyl acetal	0.69	0.69
Acetaldehyde dimethyl acetal	0.59	0.41
Acetaldehyde diethyl acetal	0.84	0.97
Propionaldehyde dimethyl acetal	0.87	0.78
Butyraldehyde dimethyl acetal	1.3_8	1.5_6
Methyl formiate	0.50	0.24
Ethyl formiate	0.75	0.43
Methyl acetate	0.82	0.43
Ethyl acetate	1.0_9	0.73
Isopropyl acetate	1.0_2	0.88
n-Propyl acetate	1.7_8	1.4_7
n-Butyl acetate	3.0_0	3.1_0
Vinyl acetate	0.97	0.73
Methyl propionate	1.2_4	0.82
Methyl acrylate	1.4_1	0.96

TABLE 47 *(Continued)*

Compound	*β,β-Hydroxydipropionitrile* V_i°, *Benzene*	*Hexaethylene glycol dimethyl ether* V_i°, *Benzene*
Methyl isobutyrate		1.0_1
Ethyl butyrate	2.3_3	2.4_7
Vinyl butyrate	2.0_8	2.3_2
Methyl butyrate		1.5
Methanol	0.91	0.53
Ethanol	1.1_3	0.73
Isopropanol	1.0_3	0.78
n-Propanol	1.9_6	1.6_3
n-Butanol	3.4_7	3.6_0
Isobutanol	2.4_4	2.5_6
sec.-Butanol	1.7_9	1.6_6
tert.-Butanol	0.8_9	
Dimethyl ether	0.07	0.03_5
Diethyl ether	0.12	0.12
Diisiopropyl ether	0.12	0.20
Di-*n*-propyl ether	0.24	0.43
Di-*n*-butyl ether	0.71	1.9_5
sym.-Hexafluorodiethyl ether		0.38
Water	2.2	1.5
Carbon disulphide	0.13	0.23
Dioxan	4.4_5	2.0_4
Tetrahydrofuran	0.91	0.58
Diacetyl		1.3

TABLE 48

R_F VALUES OF DERIVATIVES OF VOLATILE THIOALCOHOLS

(A. JACOT-GUILLARMOD AND P. CESCHINI, *Helv. Chim. Acta*, 42 (1959) 713)

Preparation of the derivatives: The mixture of thioalcohols is treated with acrolein in presence of cupric acetate. After allowing to stand for some hours the excess of acroleine is evaporated off. The 2,4-dinitrophenylhydrazones are prepared in alcoholic solutions and are isolated by filtration or by centrifugation. Their solution in chloroform is applied on the paper.

Paper: Schleicher and Schüll No. 2040 A, impregnated with a solution of dimethylformamide in alcohol (25%) and dried in air for 15 to 20 min. After applying the mixture to be separated, the paper is equilibrated at 25° for 15 h in a tank containing 2 beakers, one with dimethylformamide and the other with mobile phase. Afterwards development is started.

Solvents: Mobile phase: cyclohexane saturated with dimethylformamide at 25°. Stationary phase: dimethylformamide.

2,4-Dinitrophenylhydrazones of compounds *R-S-CH_2-CH_2-CHO* where *R* is:	R_F
CH_3	0.28
C_2H_5	0.39
C_3H_7	0.49
C_4H_9	0.57
C_5H_{11}	0.65

TABLE 49

R_F VALUES OF SOME OF THE ANALOGUES OF THYROXINE

(TERUO MATSUURA AND H. J. CAHNMANN, *J. Am. Chem. Soc.*, 81 (1959) 871)

Solvents:
- I. 1-Butanol–2*N* ammonia.
- II. 1-Butanol–dioxane–2*N* ammonia (4 : 1 : 5).
- III. tert.-Amyl alcohol–2*N* ammonia.
- IV. 1-Butanol–water.
- V. 1-Butanol–pyridine–water (12 : 1 : 12).

Paper: Whatman No. 3MM.

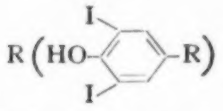
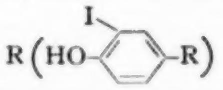
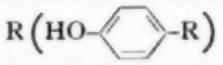
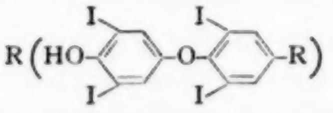
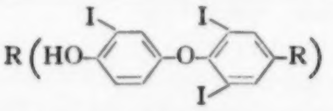
Substance	*Solvents*				
	*I*	*II*	*III*	*IV*	*V*
COOH	0.04	0.10	0.01		0.63
CH_2COOH	0.07	0.15	0.02	0.64	0.62
$(CH_2)_2COOH$	0.12	0.23	0.03	0.75	0.74
$(CH_2)_3COOH$	0.14	0.26	0.03		
$CH_2CH(CH_3)COOH$	0.15	0.28	0.05	0.85	0.77
$CH_2CH(C_6H_5)COOH$	0.25	0.35	0.10	0.85	0.83
CH = CHCOOH	0.09	0.21	0.02	0.56	0.50
$CH_2CH(OH)COOH$	0.06	0.17	0.06	0.42	0.43
$CH_2CH(NHCOCH_2Cl)COOH$	0.08	0.19	0.06		
CH(OH)COOH		0.07			0.43
COCOOH		0.10			0.3–0.73
CHO	0.83	0.79	0.38	0.94	0.93
I	0.72	0.83	0.55		
					
CH_2COOH	0.12	0.31	0.03		
$(CH_2)_2COOH$	0.21	0.33	0.12	0.75	0.74
$(CH_2)_3COOH$	0.27	0.47	0.09		
$CH_2CH(CH_3)COOH$	0.27	0.48	0.11		
$CH_2CH(C_6H_5)COOH$	0.43	0.61	0.22		
$CH_2CH(OH)COOH$	0.14	0.31	0.05		
					
CH_2COOH	0.12	0.20	0.04		
$(CH_2)_2COOH$	0.22	0.34	0.09	0.63	0.62
$(CH_2)_3COOH$	0.28	0.36	0.11		
$CH_2CH(CH_3)COOH$	0.31	0.37	0.12		
					
COOH	0.45	0.53	0.21		
CH_2COOH	0.37	0.46	0.18		
$(CH_2)_2COOH$	0.45	0.48	0.16		
$(CH_2)_3COOH$	0.44	0.54	0.20		
$CH_2CH(CH_3)COOH$	0.44	0.55	0.27		
$CH_2CH(OH)COOH$	0.33	0.46	0.22		
$CH_2CH(NHCOCH_2Cl)COOH$	0.28	0.46	0.13		
					
$(CH_2)_2COOH$	0.59	0.64	0.37		
$(CH_2)_3COOH$	0.60	0.65	0.46		

TABLE 50

R_F VALUES OF AMINO ACIDS, DISSOLVED IN ETHANOL–HYDROCHLORIC ACID

(G. ZWEIG, *Anal. Chem.*, 31 (1959) 821)

Solvent: 1-Butanol–acetic acid–water (4:1:5).
Paper: Whatman No. 3 MM.

Amino acid	*R_F value*		*Colour with ninhydrin*
	Fresh solution	*36-Hour-old solution*	
Glutamic acid	0.35	0.35	Purple
		0.60	Purple
Glutamic acid γ-ethyl ester	0.35 (minor)	0.35 (minor)	Purple
	0.60	0.60	Purple
Aspartic acid	0.28	0.28	Blue
		0.50	Yellow-brown
γ-Aminobutyric acid	0.45	0.45	Purple
		0.73	Purple
β-Alanine	0.37	0.37	Blue
		0.68	Green-blue

TABLE 51

R_F VALUES OF DEGRADATION PRODUCTS OF TYROSINE WITH DILUTE HNO_3

(H. ZAHN AND E. R. FRITZE, *Z. physiol. Chem.*, 312 (1958) 168)

Paper: Whatman No. 1.
R_F values relative to 3-nitrotyrosine = 1 are called $R_{N\text{-}tyr}$ values.

Substance	$R_{N\text{-}tyr}$
Solvent: isobutanol–2N acetic acid (100:11)	
3-Nitrotyrosine	1.0
3,5-Dinitrotyrosine	0.24
3-Nitro-4-hydroxybenzoic acid	4.5
3,5-Dinitro-4-hydroxybenzoic acid	2.0
3-Nitro-4-hydroxyphenylacetic acid	4.6
3,5-Dinitro-4-hydroxyphenylacetic acid	3.4
2,4-Dinitrophenol	4.7
Picric acid	2.5
3-Nitro-*p*-cresol	volatile
3,5-Dinitro-*p*-cresol	4.8

Substance	R_F
Solvent: butanol–2N ammonia (85:15)	
2,4-Dinitrophenol	0.61
Picric acid	0.66
3,5-Dinitro-4-hydroxybenzaldehyde	0.57

TABLE 52

R_F VALUES OF AROMATIC NITRO COMPOUNDS

(M. PERPAR, M. TIŠLER AND Ž. VRBAŠKI, *Mikrochim. Acta* (1959) 64)

Paper: Schleicher and Schüll 2043b impregnated with the petrol fraction boiling between 160 and 190° and dried in air for half an hour.
Temperature: 24°.

Substance	*Solvent*	
	Ethanol (96%) 4 *Water 3*	*Ethanol (96%) 3* *Water 5*
m-Dinitrobenzene	0.55	0.50
1,3,5-Trinitrobenzene	0.40	0.36
2,4-Dinitrotoluene		0.40
2,4,6-Trinitrotoluene		0.26
m-Nitrophenol		0.80
p-Nitrophenol		0.82
2,4-Dinitrophenol	0.75	0.84
Picric acid	0.75	0.85
Trinitro-*m*-cresol	0.90	
o-Nitraniline	0.77	0.77
m-Nitraniline	0.92	0.80
p-Nitraniline	0.86	0.85
2,4-Dinitroaniline	0.88	0.80
p-Nitrobenzoic acid	0.82	
3,5-Dinitrobenzoic acid	0.70	
1-Nitronaphthalene	0.33	0.12
1,5-Dinitronaphthalene		0.00
1,8-Dinitronaphthalene		0.58

TABLE 53

R_F AND R_G VALUES OF BENZENEPOLYCARBOXYLIC ACIDS

(J. E. GERMAIN, J. MONTREUIL AND P. KOUKOS, *Bull. soc. chim. France*, (1959) 115)

Solvents: I. Phenol–isopropanol–formic acid. Paper: Whatman No. 1.
IIa. Ethanol–ammonia (70:30). Paper: Whatman No. 3.
IIb. Ethanol–ammonia (50:50). Paper: Whatman No. 3.
III. Butanol–acetic acid–water (4:1:5). Paper: Whatman No. 3 or 4.
Temperature: room temperature (about 16°).

Acids	*Solvents*			
	I (R_F)	*IIa* (R_G)	*IIb* (R_G)	*III* (R_F)
Benzenemonocarboxylic	0.85–0.90	1.50–1.70		0.88–0.92
1,4-Benzenedicarboxylic	0.80–0.81	1.13–1.20		0.83–0.88
1,3-Benzenedicarboxylic	0.80–0.81	1.13–1.20		0.85–0.89
1,2-Benzenedicarboxylic	0.80–0.81	1		0.75–0.79
1,3,5-Benzenetricarboxylic	0.65–0.67	0.30–0.50	1	0.82–0.88
1,2,4-Benzenetricarboxylic	0.65–0.67	0.25–0.42	0.90	0.74–0.80
1,2,3-Benzenetricarboxylic	0.65–0.67	0.17–0.34	0.80–0.84	0.71–0.74
1,2,4,6-Benzenetetracarboxylic	0.48–0.50		0.72–0.78	0.59–0.62
1,2,4,5-Benzenetetracarboxylic	0.48–0.50		0.68–0.74	0.30–0.37
1,2,3,4-Benzenetetracarboxylic	0.48–0.50		0.55–0.60	0.26–0.30
Benzenepentacarboxylic	0.30–0.34		0.30–0.40	0.18–0.24
Benzenehexacarboxylic	0.18–0.22		0.0	0.09–0.12

TABLE 54

VALUES OF THE CORRECTION FACTOR j OF JAMES AND MARTIN USED IN GAS CHROMATOGRAPHY, FOR VALUES OF THE RATIO OF INLET TO OUTLET PRESSURE BETWEEN 1 AND 3

The Gas Chromatographic Section of the Group for the Advancement of Spectrographic and Physicochemical Methods of Analysis* has compiled, for the use of its members, a table of values for the correction factor j of JAMES AND MARTIN:

$$j = \frac{3}{2}\,\frac{a^2 - 1}{a^3 - 1}$$

for values of the ratio of inlet to outlet pressure:

$$a = p_i/p_o$$

between $a = 1$ and $a = 3$, at intervals of 0.001.

Since these data could be of value to many workers using gas chromatography, the G.A.M.S. has decided to publish the table. The help given by the Section of Applied Mathematics of the Esso Standard Co., in calculating the values is gratefully acknowledged.

a	j	a	j	a	j	a	j
		1.020	0.99006	1.040	0.98026	1.060	0.97059
1.001	0.99950	1	0.98957	1	0.97977	1	011
2	900	2	908	2	929	2	0.96963
3	850	3	858	3	880	3	916
4	800	4	809	4	832	4	868
5	750	5	760	5	783	5	820
6	700	6	711	6	735	6	772
7	650	7	662	7	686	7	724
8	601	8	613	8	638	8	676
9	551	9	564	9	589	9	629
1.010	501	1.030	515	1.050	541	1.070	581
1	452	1	466	1	493	1	533
2	402	2	417	2	445	2	486
3	352	3	368	3	396	3	438
4	303	4	319	4	348	4	391
5	253	5	270	5	300	5	343
6	204	6	221	6	252	6	296
7	154	7	172	7	204	7	248
8	105	8	124	8	156	8	201
9	056	9	075	9	107	9	153

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* Groupement pour l'Avancement des Méthodes Spectrographiques et physiochimiques d'analyses (G.A.M.S.).

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
1.080	0.96106	1.120	0.94238	1.160	0.92423	1.200	0.90659
1	059	1	192	1	378	1	615
2	011	2	146	2	334	2	572
3	0.95964	3	101	3	289	3	529
4	917	4	055	4	244	4	485
5	870	5	009	5	200	5	442
6	822	6	0.93963	6	155	6	399
7	775	7	917	7	111	7	355
8	728	8	871	8	066	8	312
9	681	9	825	9	022	9	269
1.090	634	1.130	780	1.170	0.91977	1.210	226
1	587	1	734	1	933	1	183
2	540	2	688	2	888	2	139
3	493	3	643	3	844	3	096
4	446	4	597	4	800	4	053
5	400	5	552	5	756	5	010
6	353	6	506	6	711	6	0.89967
7	306	7	461	7	667	7	924
8	259	8	415	8	623	8	881
9	212	9	370	9	579	9	838
1.100	166	1.140	324	1.180	535	1.220	796
1	119	1	279	1	491	1	753
2	072	2	234	2	446	2	710
3	026	3	188	3	402	3	667
4	0.94979	4	143	4	358	4	624
5	933	5	098	5	314	5	582
6	886	6	053	6	271	6	539
7	840	7	007	7	227	7	496
8	793	8	0.92962	8	183	8	454
9	747	9	917	9	139	9	411
1.110	700	1.150	872	1.190	095	1.230	369
1	654	1	827	1	051	1	326
2	608	2	782	2	008	2	284
3	561	3	737	3	0.90964	3	241
4	515	4	692	4	920	4	199
5	469	5	647	5	877	5	156
6	423	6	602	6	833	6	114
7	377	7	557	7	789	7	072
8	331	8	513	8	746	8	029
9	285	9	468	9	702	9	0.88987

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
1.240	0.88945	1.280	0.87280	1.320	0.85663	1.360	0.84093
1	903	1	239	1	623	1	054
2	860	2	198	2	584	2	016
3	818	3	157	3	544	3	0.83977
4	776	4	116	4	504	4	938
5	734	5	075	5	464	5	900
6	692	6	034	6	425	6	861
7	650	7	0.86994	7	385	7	823
8	608	8	953	8	345	8	784
9	566	9	912	9	306	9	746
1.250	524	1.290	871	1.330	266	1.370	708
1	482	1	831	1	227	1	669
2	440	2	790	2	187	2	631
3	398	3	749	3	148	3	593
4	357	4	709	4	108	4	554
5	315	5	668	5	069	5	516
6	273	6	628	6	030	6	478
7	231	7	587	7	0.84990	7	440
8	190	8	547	8	951	8	401
9	148	9	506	9	912	9	363
1.260	106	1.300	466	1.340	872	1.380	325
1	065	1	425	1	833	1	287
2	023	2	385	2	794	2	249
3	0.87982	3	345	3	755	3	211
4	940	4	304	4	716	4	173
5	899	5	264	5	676	5	135
6	857	6	224	6	637	6	097
7	816	7	183	7	598	7	059
8	774	8	143	8	559	8	021
9	733	9	103	9	520	9	0.82983
1.270	692	1.310	063	1.350	481	1.390	945
1	650	1	023	1	442	1	907
2	609	2	0.85983	2	403	2	870
3	568	3	943	3	364	3	832
4	527	4	903	4	326	4	794
5	485	5	863	5	287	5	756
6	444	6	823	6	248	6	719
7	403	7	783	7	209	7	681
8	362	8	743	8	170	8	643
9	321	9	703	9	132	9	606

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
1.400	0.82568	1.440	0.81088	1.480	0.79650	1.520	0.78254
1	531	1	051	1	615	1	220
2	493	2	015	2	579	2	185
3	456	3	0.80978	3	544	3	151
4	418	4	942	4	509	4	117
5	381	5	906	5	473	5	082
6	343	6	869	6	438	6	048
7	306	7	833	7	403	7	014
8	269	8	797	8	368	8	0.77980
9	231	9	761	9	332	9	945
1.410	194	1.450	724	1.490	297	1.530	911
1	157	1	688	1	262	1	877
2	120	2	652	2	227	2	843
3	082	3	616	3	192	3	809
4	045	4	580	4	157	4	775
5	008	5	544	5	122	5	741
6	0.81971	6	508	6	087	6	707
7	934	7	472	7	052	7	673
8	897	8	436	8	017	8	639
9	860	9	400	9	0.78982	9	605
1.420	823	1.460	364	1.500	947	1.540	571
1	786	1	328	1	912	1	537
2	749	2	292	2	877	2	503
3	712	3	256	3	842	3	469
4	675	4	220	4	807	4	436
5	638	5	184	5	773	5	402
6	601	6	148	6	738	6	368
7	564	7	113	7	703	7	334
8	527	8	077	8	668	8	301
9	491	9	041	9	634	9	267
1.430	454	1.470	006	1.510	599	1.550	233
1	417	1	0.79970	1	564	1	200
2	380	2	934	2	530	2	166
3	344	3	899	3	495	3	132
4	307	4	863	4	461	4	099
5	270	5	827	5	426	5	065
6	234	6	792	6	392	6	032
7	197	7	756	7	357	7	0.76998
8	161	8	721	8	323	8	965
9	124	9	685	9	288	9	931

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
1.560	0.76898	1.600	0.75581	1.640	0.74302	1.680	0.73059
1	865	1	548	1	270	1	028
2	831	2	516	2	239	2	0.72997
3	798	3	484	3	207	3	967
4	764	4	451	4	176	4	936
5	731	5	419	5	144	5	906
6	698	6	387	6	113	6	875
7	665	7	354	7	081	7	845
8	631	8	322	8	050	8	814
9	598	9	290	9	019	9	784
1.570	565	1.610	258	1.650	0.73987	1.690	753
1	532	1	225	1	956	1	723
2	499	2	193	2	925	2	693
3	466	3	161	3	894	3	662
4	433	4	129	4	862	4	632
5	400	5	097	5	831	5	602
6	367	6	065	6	800	6	571
7	333	7	033	7	769	7	541
8	301	8	001	8	738	8	511
9	268	9	0.74969	9	707	9	481
1.580	235	1.620	937	1.660	676	1.700	450
1	202	1	905	1	644	1	420
2	169	2	873	2	613	2	390
3	136	3	841	3	582	3	360
4	103	4	809	4	551	4	330
5	070	5	777	5	520	5	300
6	038	6	745	6	489	6	270
7	005	7	713	7	459	7	239
8	0.75972	8	681	8	428	8	209
9	939	9	650	9	397	9	179
1.590	907	1.630	618	1.670	366	1.710	149
1	874	1	586	1	335	1	119
2	841	2	554	2	304	2	090
3	809	3	523	3	273	3	060
4	776	4	491	4	243	4	030
5	743	5	459	5	212	5	000
6	711	6	428	6	181	6	0.71970
7	678	7	396	7	151	7	940
8	646	8	365	8	120	8	910
9	613	9	333	9	089	9	880

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
1.720	0.71851	1.760	0.70677	1.800	0.69536	1.840	0.68427
1	821	1	648	1	508	1	399
2	791	2	619	2	480	2	372
3	762	3	590	3	452	3	345
4	732	4	561	4	424	4	317
5	702	5	533	5	396	5	290
6	673	6	504	6	368	6	263
7	643	7	475	7	340	7	236
8	613	8	446	8	312	8	208
9	584	9	417	9	284	9	181
1.730	554	1.770	389	1.810	256	1.850	154
1	525	1	360	1	228	1	127
2	495	2	331	2	200	2	100
3	466	3	303	3	172	3	073
4	436	4	274	4	144	4	046
5	407	5	245	5	116	5	019
6	377	6	217	6	088	6	0.67992
7	348	7	188	7	061	7	965
8	318	8	159	8	033	8	938
9	289	9	131	9	005	9	911
1.740	260	1.780	102	1.820	0.68977	1.860	884
1	230	1	074	1	950	1	857
2	201	2	045	2	922	2	830
3	172	3	017	3	894	3	803
4	142	4	0.69988	4	867	4	776
5	113	5	960	5	839	5	749
6	084	6	932	6	811	6	722
7	055	7	903	7	784	7	695
8	026	8	875	8	756	8	668
9	0.70996	9	846	9	729	9	642
1.750	967	1.790	818	1.830	701	1.870	615
1	938	1	790	1	674	1	588
2	909	2	762	2	646	2	561
3	880	3	733	3	619	3	535
4	851	4	705	4	591	4	508
5	822	5	677	5	564	5	481
6	793	6	649	6	536	6	454
7	764	7	620	7	509	7	428
8	735	8	592	8	481	8	401
9	706	9	564	9	454	9	375

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
1.880	0.67348	1.920	0.66299	1.960	0.65278	2.000	0.64285
1	321	1	273	1	253	1	261
2	295	2	247	2	228	2	236
3	268	3	221	3	203	3	212
4	242	4	196	4	178	4	187
5	215	5	170	5	153	5	163
6	189	6	144	6	128	6	139
7	162	7	118	7	103	7	114
8	136	8	092	8	077	8	090
9	109	9	067	9	052	9	065
1.890	083	1.930	041	1.970	027	2.010	041
1	057	1	015	1	002	1	017
2	030	2	0.65990	2	0.64977	2	0.63993
3	004	3	964	3	953	3	968
4	0.66977	4	938	4	928	4	944
5	951	5	913	5	903	5	920
6	925	6	887	6	878	6	895
7	899	7	862	7	853	7	871
8	872	8	836	8	828	8	847
9	846	9	811	9	803	9	823
1.900	820	1.940	785	1,980	778	2.020	799
1	794	1	760	1	754	1	775
2	767	2	734	2	729	2	750
3	741	3	709	3	704	3	726
4	715	4	683	4	679	4	702
5	689	5	658	5	654	5	678
6	663	6	632	6	630	6	654
7	637	7	607	7	605	7	630
8	611	8	581	8	580	8	606
9	584	9	556	9	556	9	582
1.910	558	1.950	531	1.990	531	2.030	558
1	532	1	505	1	506	1	534
2	506	2	480	2	482	2	510
3	480	3	455	3	457	3	486
4	454	4	430	4	432	4	462
5	428	5	404	5	408	5	438
6	402	6	379	6	383	6	414
7	377	7	354	7	359	7	390
8	351	8	329	8	334	8	366
9	325	9	303	9	310	9	343

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
2.040	0.63319	2.080	0.62378	2.120	0.61462	2.160	0.60570
1	295	1	355	1	439	1	548
2	271	2	332	2	417	2	526
3	247	3	308	3	394	3	504
4	224	4	285	4	372	4	482
5	200	5	262	5	349	5	460
6	176	6	239	6	327	6	438
7	152	7	216	7	304	7	416
8	129	8	193	8	282	8	394
9	105	9	170	9	259	9	372
2.050	081	2.090	147	2.130	237	2.170	351
1	058	1	124	1	214	1	329
2	034	2	101	2	192	2	307
3	010	3	078	3	169	3	285
4	0.62987	4	055	4	147	4	263
5	963	5	032	5	125	5	241
6	939	6	009	6	102	6	220
7	916	7	0.61986	7	080	7	198
8	892	8	963	8	058	8	176
9	869	9	940	9	035	9	154
2.060	845	2.100	917	2.140	013	2.180	133
1	822	1	894	1	0.60991	1	111
2	798	2	871	2	968	2	089
3	775	3	848	3	946	3	068
4	751	4	825	4	924	4	046
5	728	5	803	5	902	5	024
6	704	6	780	6	879	6	003
7	681	7	757	7	857	7	0.59981
8	658	8	734	8	835	8	959
9	634	9	711	9	813	9	938
2.070	611	2.110	689	2.150	791	2.190	916
1	587	1	666	1	769	1	895
2	564	2	643	2	746	2	873
3	541	3	621	3	724	3	851
4	517	4	598	4	702	4	830
5	494	5	575	5	680	5	808
6	471	6	552	6	658	6	787
7	448	7	530	7	636	7	765
8	424	8	507	8	614	8	744
9	401	9	485	9	592	9	722

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
2.200	0.59701	2.240	0.58854	2.280	0.58029	2.320	0.57225
1	680	1	833	1	009	1	205
2	658	2	813	2	0.57989	2	185
3	637	3	792	3	968	3	166
4	615	4	771	4	948	4	146
5	594	5	750	5	928	5	126
6	573	6	729	6	907	6	106
7	551	7	708	7	887	7	086
8	530	8	688	8	867	8	067
9	509	9	667	9	847	9	047
2.210	487	2.250	646	2.290	826	2.330	027
1	466	1	625	1	806	1	007
2	445	2	605	2	786	2	0.56988
3	423	3	584	3	766	3	968
4	402	4	563	4	745	4	948
5	381	5	542	5	725	5	929
6	360	6	522	6	705	6	909
7	338	7	501	7	685	7	889
8	317	8	480	8	665	8	870
9	296	9	460	9	645	9	850
2.220	275	2.260	439	2.300	625	2.340	831
1	254	1	419	1	605	1	811
2	233	2	398	2	584	2	791
3	212	3	377	3	564	3	772
4	190	4	357	4	544	4	752
5	169	5	336	5	524	5	733
6	148	6	316	6	504	6	713
7	127	7	295	7	484	7	694
8	106	8	275	8	464	8	674
9	085	9	254	9	444	9	655
2.230	064	2.270	234	2.310	424	2.350	635
1	043	1	213	1	404	1	616
2	022	2	193	2	384	2	596
3	001	3	172	3	364	3	577
4	0.58980	4	152	4	344	4	557
5	959	5	131	5	325	5	538
6	938	6	111	6	305	6	519
7	917	7	090	7	285	7	499
8	896	8	070	8	265	8	480
9	875	9	050	9	245	9	460

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
2.360	0.56441	2.400	0.55676	2.440	0.54931	2.480	0.54203
1	422	1	657	1	912	1	185
2	402	2	639	2	894	2	167
3	383	3	620	3	875	3	149
4	364	4	601	4	857	4	131
5	344	5	582	5	839	5	113
6	325	6	563	6	820	6	095
7	306	7	544	7	802	7	077
8	287	8	526	8	784	8	059
9	267	9	507	9	765	9	042
2.370	248	2.410	488	2.450	747	2.490	024
1	229	1	469	1	729	1	006
2	210	2	451	2	710	2	0.53988
3	190	3	432	3	692	3	970
4	171	4	413	4	674	4	952
5	152	5	394	5	656	5	935
6	133	6	376	6	637	6	917
7	114	7	357	7	619	7	899
8	095	8	338	8	601	8	881
9	075	9	320	9	583	9	863
2.380	056	2.420	301	2.460	564	2.500	846
1	037	1	282	1	546	1	828
2	018	2	264	2	528	2	810
3	0.55999	3	245	3	510	3	792
4	980	4	227	4	492	4	775
5	961	5	208	5	474	5	757
6	942	6	189	6	456	6	739
7	923	7	171	7	437	7	722
8	904	8	152	8	419	8	704
9	885	9	134	9	401	9	686
2.390	866	2.430	115	2.470	383	2.510	669
1	847	1	097	1	365	1	651
2	828	2	078	2	347	2	633
3	809	3	060	3	329	3	616
4	790	4	041	4	311	4	598
5	771	5	023	5	293	5	581
6	752	6	004	6	275	6	563
7	733	7	0.54986	7	257	7	545
8	714	8	967	8	239	8	528
9	695	9	949	9	221	9	510

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
2.520	0.53493	2.560	0.52800	2.600	0.52123	2.640	0.51462
1	475	1	783	1	106	1	446
2	458	2	765	2	090	2	430
3	440	3	748	3	073	3	413
4	423	4	731	4	056	4	397
5	405	5	714	5	040	5	381
6	388	6	697	6	023	6	365
7	370	7	680	7	006	7	348
8	353	8	663	8	0.51990	8	332
9	335	9	646	9	973	9	316
2.530	318	2.570	629	2.610	956	2.650	300
1	301	1	612	1	940	1	283
2	283	2	595	2	923	2	267
3	266	3	578	3	907	3	251
4	248	4	561	4	890	4	235
5	231	5	544	5	873	5	219
6	214	6	527	6	857	6	202
7	196	7	510	7	840	7	186
8	179	8	493	8	824	8	170
9	161	9	476	9	807	9	154
2.540	144	2.580	459	2.620	791	2.660	138
1	127	1	442	1	774	1	122
2	110	2	426	2	758	2	106
3	092	3	409	3	741	3	089
4	075	4	392	4	725	4	073
5	058	5	375	5	708	5	057
6	040	6	358	6	692	6	041
7	023	7	341	7	675	7	025
8	006	8	324	8	659	8	009
9	0.52989	9	308	9	642	9	0.50993
2.550	971	2.590	291	2.630	626	2.670	977
1	954	1	274	1	610	1	961
2	937	2	257	2	593	2	945
3	920	3	240	3	577	3	929
4	903	4	224	4	560	4	913
5	885	5	207	5	544	5	897
6	868	6	190	6	528	6	881
7	851	7	173	7	511	7	865
8	834	8	156	8	495	8	849
9	817	9	140	9	479	9	833

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
2.680	0.50817	2.720	0.50187	2.760	0.49571	2.800	0.48969
1	801	1	171	1	555	1	954
2	785	2	155	2	540	2	939
3	769	3	140	3	525	3	924
4	753	4	124	4	510	4	909
5	737	5	109	5	495	5	894
6	721	6	093	6	479	6	879
7	706	7	078	7	464	7	865
8	690	8	062	8	449	8	850
9	674	9	047	9	434	9	835
2.690	658	2.730	031	2.770	419	2.810	820
1	642	1	016	1	404	1	805
2	626	2	000	2	389	2	791
3	610	3	0.49985	3	373	3	776
4	595	4	969	4	358	4	761
5	579	5	954	5	343	5	746
6	563	6	938	6	328	6	732
7	547	7	923	7	313	7	717
8	531	8	908	8	298	8	702
9	516	9	892	9	283	9	687
2.700	500	2.740	877	2.780	268	2.820	673
1	484	1	861	1	253	1	658
2	468	2	846	2	238	2	643
3	453	3	831	3	223	3	629
4	437	4	815	4	208	4	614
5	421	5	800	5	193	5	599
6	406	6	785	6	178	6	585
7	390	7	769	7	163	7	570
8	374	8	754	8	148	8	555
9	358	9	739	9	133	9	541
2.710	343	2.750	723	2.790	118	2.830	526
1	327	1	708	1	103	1	511
2	311	2	693	2	088	2	497
3	296	3	677	3	073	3	482
4	280	4	662	4	058	4	468
5	265	5	647	5	043	5	453
6	249	6	632	6	028	6	438
7	233	7	616	7	013	7	424
8	218	8	601	8	0.48998	8	409
9	202	9	586	9	983	9	395

a	*j*	*a*	*j*	*a*	*j*	*a*	*j*
2.840	0.48380	2.880	0.47805	2.920	0.47242	2.960	0.46692
1	366	1	791	1	228	1	678
2	351	2	776	2	214	2	665
3	336	3	762	3	200	3	651
4	322	4	748	4	186	4	637
5	307	5	734	5	173	5	624
6	293	6	720	6	159	6	610
7	278	7	705	7	145	7	597
8	264	8	691	8	131	8	583
9	250	9	677	9	117	9	570
2.850	235	2.890	663	2.930	103	2.970	556
1	221	1	649	1	090	1	543
2	206	2	635	2	076	2	529
3	192	3	620	3	062	3	515
4	177	4	606	4	048	4	502
5	163	5	592	5	034	5	488
6	148	6	578	6	020	6	475
7	134	7	564	7	007	7	461
8	120	8	550	8	0.46993	8	448
9	105	9	536	9	979	9	435
2.860	091	2.900	522	2.940	965	2.980	421
1	076	1	508	1	952	1	408
2	062	2	494	2	938	2	394
3	048	3	480	3	924	3	381
4	033	4	466	4	910	4	367
5	019	5	452	5	897	5	354
6	005	6	438	6	883	6	340
7	0.47990	7	424	7	869	7	327
8	976	8	410	8	856	8	314
9	962	9	396	9	842	9	300
2.870	947	2.910	382	2.950	828	2.990	287
1	933	1	368	1	815	1	273
2	919	2	354	2	801	2	260
3	904	3	340	3	787	3	247
4	890	4	326	4	774	4	233
5	876	5	312	5	760	5	220
6	862	6	298	6	746	6	207
7	847	7	284	7	733	7	193
8	833	8	270	8	719	8	180
9	819	9	256	9	705	9	167
						3.000	153

TABLE 55

R_F VALUES OF 2,4-DINITROPHENYLHYDRAZONES OF 2-ALKANONES, ALK-2-ENALS, AND ALKA-2,4-DIENALS

(A. M. GADDIS AND R. ELLIS, *Anal. Chem.*, 31 (1959) 870)

System 1: Paper: Whatman No. 3 paper impregnated with 20% propylene glycol–80% methanol (v/v) and dried.
Solvent: 96% Skellysolve–4% methanol (v/v).
System 2: Paper: Whatman No. 3 paper impregnated with a 7% solution of vaseline in Skellysolve and dried.
Solvent: 89% methanol–11% water (v/v).

2,4-Dinitrophenylhydrazone of	*R_F values in*	
	System 1	*System 2*
2-Alkanones		
C_3	0.63	
C_4	0.81	
C_5	0.94	
C_6		0.72
C_7		0.65
C_8		0.59
C_9		0.51
C_{11}		0.36
C_{13}		0.24
Alk-2-enals		
C_3	0.38	
C_4	0.49	
C_5	0.67	
C_6	0.80	
C_7	0.92	0.76
C_8		0.70
C_9		0.65
C_{10}		0.57
C_{11}		0.49
C_{12}		0.42
Alka-2,4-dienals		
C_5	0.45	
C_3	0.56	
C_7	0.73	
C_8	0.85	
C_9	0.94	0.67
C_{10}		0.61
C_{11}		0.54
C_{12}		0.47

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